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(54) Title: METHODS AND REAGENTS FOR HLA DRBETA DNA TYPING

Patterns	Probes																					
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
DRB1*0101/0102	+																					
DRB1*0103	+																					
DRB1*1516	+																					
DRB1*0301																						
Also possibly Heterozygous: DRB1 alleles 0301 & 0302, 0301 & 1403																						
DRB1*0302																						
Also possibly Heterozygous: DRB1 alleles 0302 & 1403																						
DRB1*0401																						
DRB1*0402																						
DRB1*0403/0406/0407																						
DRB1*0404/0408																						
DRB1*0405/0410																						
Also possibly Heterozygous: DRB1 alleles 0404/0408 & 0405/0410																						
DRB1*0409																						
Also possibly Heterozygous: DRB1 alleles 0401 & 0409																						

(57) Abstract

Primers for amplification of specific nucleic acid sequences of the second exon of HLA DRbeta genes and probes for identifying polymorphic sequences contained in the amplified DNA can be used in processes for typing homozygous or heterozygous samples from a variety of sources and for detecting allelic variants non-distinguishable by serological methods. This HLA

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Methods and Reagents for HLA DRbeta DNA Typing

Cross-Reference to Related Applications

This application is a continuation-in-part of copending Serial No. 623,098, filed December 6, 1990, which is a continuation-in-part of copending Serial No. 491,210, filed August 15, 1989, which is a continuation of now abandoned Serial No. 899,344, filed August 22, 1986, which is a continuation-in-part of now abandoned Serial No. 839,331, filed March 13, 1986, each of which is incorporated herein by reference.

Background of the Invention

Field of the Invention

10 The present invention provides methods and reagents for DNA typing HLA DRbeta (DRB) nucleic acids. The invention enables one to type homozygous or heterozygous samples from a variety of sources, including samples comprising RNA or cDNA templates, and to detect allelic variants not distinguishable by present serological, cellular, or biochemical methods. The present typing system facilitates
15 typing tissue for transplantation, determining individual identity, and identifying disease susceptible individuals. The invention therefore has applications in the field of medicine generally and medical research and diagnostics specifically, the field of forensic science, and the field of molecular biology.

Description of Related Art

20 The HLA Class II proteins HLA DR, HLA DQ, and HLA DP are encoded by genes in the major histocompatibility complex (MHC) region on the short arm of human chromosome 6. The Class II proteins are heterodimeric glycoproteins consisting of an approximately 34 kD alpha chain and an approximately 29 kD beta chain. The Class II proteins are expressed on the cell surface of macrophages, B-cells,
25 activated T-cells, and other cell types and are involved in binding and presenting antigen to helper T-lymphocytes. See the article entitled "Structure, function, and genetics of the Human Class II molecules" by Giles and Capra, 1985, Adv. Immunol. 37:1. In addition, the Class II proteins influence specific immune responsiveness by determining the repertoire of expressed T-cell receptors in mature T-lymphocytes. For
30 a general review of the HLA Class II genes and proteins, see the article entitled "Structure, sequence and polymorphism in the HLA D region" by Trowsdale *et al.*, 1985, Immunol. Rev. 85:5.

35 The Class II alpha and beta chains are encoded by separate genes, and the DP, DQ, and, DR genes are located in separate regions of the MHC. In the DR region, a single DRA locus, or gene, encodes the non-polymorphic DRalpha chain, but five

different DRB loci, termed DRB1, DRB2 (now known as DRB6), DRB3, DRB4, and DRB5, encode the polymorphic DRbeta chain. Some loci are present only on certain haplotypes (such as DRB5 on the DR2 haplotype); in addition, the number of expressed DRB genes also varies between haplotypes. See the article entitled "HLA-
5 DRbeta genes vary in number between different DR-specificities, whereas the number of DQbeta genes is constant" by Bohme *et al.*, 1985, *J. Immunol.* 135:2149.

The number of distinct DRB1 alleles identified is continually increasing. The 1989 report from the WHO Nomenclature Committee for factors of the HLA system identified 34 distinct DRB1 alleles; these alleles are thought to express the serological
10 DR specificities DR1 to DRw18. (see the article entitled "Nomenclature for factors of the HLA system, 1989" by the WHO Nomenclature Committee, 1990, *Immunogenetics* 31:131-140, incorporated herein by reference). By the 1990 report, the number of DRB1 alleles recognized had risen to 45 (see the article entitled "Nomenclature for factors of the HLA system, 1990" by the WHO Nomenclature
15 Committee, 1991, *Immunogenetics* 33:301-309, incorporated herein by reference). The present invention provides the sequences of several newly discovered alleles.

The alleles of the DRB2 locus (now termed the DRB6 locus), which are present on DR1, DR2, and DRw10 haplotypes, are apparently not expressed. See the article
20 entitled "Analysis of isotypic and allotypic sequence variation in the HLA DRB region using the *in vitro* enzymatic amplification of specific DNA segments" by Erlich *et al.*, 1989, in *Immunobiology of HLA* (Dupont ed., Springer-Verlag, New York).

The alleles of the DRB3 locus, which is thought to encode the supertypic specificity DRw52 (DRw52a, DRw52b, and DRw52c), are present on the DR3, DRw6, DRw11, DRw12, DRw13, DRw14, DRw17, and DRw18 haplotypes.
25

The DRB4 locus, which has a single allele, encodes the DRw53 supertypic specificity and is present only on the DR4, DR7, and DRw9 haplotypes. See the article
entitled "Structural relationships between the DRbeta1 and DRbeta2 subunits in DR4, 7, and w9 haplotypes and the DRw53 (MT3) specificity" by Matsuyama *et al.*, 1986, *J. Immunol.* 137:934.
30

The alleles of the DRB5 locus are present only on DR2 haplotypes. See the article entitled "Analysis of isotypic and allotypic sequence variation in the HLA DRB region using the *in vitro* enzymatic amplification of specific DNA segments," *supra*.

Polymorphism of the Class II DR antigens (proteins) is currently typed with allosera obtained from multiparous women in a microcytotoxicity assay on purified B
35 lymphocytes. In addition, cellular typing protocols capable of greater specificity and based on either the specificity of alloreactive T-cell clones or the proliferative response of T-cell cultures to stimulation by homozygous typing cells (HTCs) have been developed.

These cellular-based analyses define the Dw specificities that further subdivide many of the serologically defined antigens, e.g., the five Dw subtypes of DR4. See the article entitled "Sequence polymorphism of HLA DRbeta I alleles relating to T cell recognized determinants" by Cairns *et al.*, 1985, *Nature* 317:166. Both the serological and cellular assay procedures, however, are difficult and time-consuming. HLA DR DNA typing protocols based on restriction fragment length polymorphisms (RFLP) have also been developed. See U.S. Patent No. 4,582,788, incorporated herein by reference. However, these RFLP-based analyses require large amounts of high molecular weight DNA, are labor intensive, and the limited number of informative restriction enzymes in turn limits the results obtained.

The advent of the polymerase chain reaction (PCR) has facilitated the analysis and manipulation of complex genomic DNA. The PCR process enables one to amplify a specific sequence of nucleic acid starting from a very complex mixture of nucleic acids and is more fully described in U.S. Patent Nos. 4,683,195; 4,683,202; 4,889,818; and 4,965,188, and European Patent Publication Nos. 237,362 and 258,017, each of which is incorporated herein by reference.

The PCR process has also facilitated typing the Class II HLA DNA of an individual. Scientists have studied the polymorphic second exon of DRB loci in genomic DNA by designing oligonucleotide primers and using those primers to amplify the sequences of interest. See the article entitled "Sequence analysis of the HLA DRB and HLA DQB loci from three *Pemphigus vulgaris* patients" by Scharf *et al.*, 1988, *Hum. Immunol.* 22:61.

When the PCR primers contain restriction enzyme recognition sequences, the amplified DNA can be cloned directly into sequencing vectors, and the nucleotide sequence of the amplification product can be readily determined. See the article entitled "Direct cloning and sequence analysis of enzymatically amplified genomic sequences" by Scharf *et al.*, 1986, *Hum. Immunol.* 233:1076.

The amplified DNA can also be studied by detection methods that employ sequence-specific oligonucleotide (SSO) probes. See the article entitled "Analysis of enzymatically amplified beta-globin and HLA DQalpha DNA with allele-specific oligonucleotide probes" by Saiki *et al.*, 1986, *Nature* 324:163.

Despite these advances, the complexity of the HLA DRbeta genes has prevented the development of an informative and efficient means for determining the HLA DRbeta DNA type of an individual. The present invention meets the need for an efficient, informative DRbeta DNA typing method by providing novel processes and reagents. These novel processes and reagents have in turn led to the discovery of previously unknown DRbeta alleles, which can also be typed and identified by the present method.

The present typing system can be used to type cDNA synthesized from mRNA and to type and study the expression of DRB genes in tissues, transgenic systems, disease states, and cells lines. Cells that do not express the DR antigens or show unusual seroreactivity, such as tumor cells, can be readily typed. Moreover, samples
5 from unusual sources, e.g., ancient DNAs or forensic samples, can be typed, even when the DNA sample is degraded or when only very small quantities are available for analysis.

Because PCR can amplify a fragment of target DNA over a million-fold, and because the present system can employ PCR-generated nucleic acid, radioactively
10 labeled probes are not necessary, and nonisotopic SSOs covalently coupled to horseradish peroxidase (HRP) provide sufficient sensitivity for detection. The presence of the specifically bound HRP-labeled probes of the invention can be detected in a simple dot-blot format by chromogenic dye or chemiluminescent substrates in a matter of minutes.

15 Summary of the Invention

The present invention provides amplification and detection methods, generic and allele or group specific amplification primers, nonisotopic sequence specific oligonucleotide (SSO) probes, including probes for identifying previously unknown alleles at the DRB1 locus, and kits for practicing the methods, that together provide a
20 rapid, simple and precise system for typing the HLA DRB alleles, including those that cannot be distinguished by serological methods.

In one aspect, the present invention provides a method for determining the DRbeta DNA type of nucleic acid in a sample, which method comprises (a) amplifying any nucleic acids in the sample that contain a DRbeta gene second exon; (b) hybridizing
25 said nucleic acid amplified in step (a) to a first panel of oligonucleotide probes under conditions such that said probes hybridize only to exactly complementary sequences greater than ten nucleotides in length; (c) amplifying a specific subset of nucleic acids in the sample that contain DRbeta gene second exon sequences; (d) hybridizing said nucleic acid amplified in step (c) with a second panel of oligonucleotide probes under
30 conditions such that said probes hybridize only to exactly complementary sequences greater than ten nucleotides in length; and (e) determining from the pattern of probe hybridization in steps (b) and (d) the DRbeta alleles from which the DRbeta DNA in said sample originates.

In another aspect, the present invention provides a method for determining
35 whether a sample comprises nucleic acid from the DRB1 allele of a serological type selected from the group consisting of the DR1, DR2, DR4, DR7, DRw9, and DRw10 types, which method comprises (a) amplifying the DRB1 nucleic acid in the sample;

and (b) hybridizing said nucleic acid amplified in step (a) to a panel of oligonucleotide probes under conditions such that said probes hybridize only to exactly complementary sequences greater than ten nucleotides in length, and wherein said probes hybridize to unique polymorphic sequences encoding amino acid residues 9 to 16 of the DRB1
5 second exon.

Brief Description of the Figures

Figure 1 shows the results of DRB generic and DRB1 specific amplification and detection; see Example 2.

10 Figure 2 shows the results of DRB1 specific amplification and detection; see Example 2.

Figure 3 shows the results of HLA DRB DNA typing of the classical serological types DR1 through DR10; see Example 3.

Figure 4 shows the results of HLA DRB DNA typing to subtype cell lines with the DR4 serological specificity; see Example 5.

15 Figure 5 shows the results of HLA DRB DNA typing to subtype the DR3, DR5, DRw6, and DRw8 specificities; see Example 6.

Figure 6 shows the tabulation of probe hybridization results to determine DRB type; see Example 6.

20 Figure 7 shows the results of HLA DRB DNA typing of a number of different cell lines; see Examples 6 and 7.

Figure 8 shows the HLA DRB DNA subtyping of DR3 cell lines; see Example 7.

Figure 9 shows the tabulation of probe hybridization results to determine the HLA DRB DNA type of heterozygous and other unusual samples; see Example 7.

25 Figure 10 shows the tabulation of probe hybridization results to determine the HLA DRB DNA type; see Example 9.

Figure 11, 12, and 13 show the tabulation of probe hybridization results to determine DRB allele type; see Example 9.

Detailed Description of the Invention

30 The present invention provides an HLA DRB typing system and sequence specific oligonucleotide probes (SSOs) for analyzing DRB alleles. The invention can be used to type heterozygous samples from a variety of sources, including cDNA templates, and can be used to detect allelic variants not distinguishable by serological methods. This typing system can utilize a dot-blot format that is simple and rapid to
35 perform, produces detectable signals in minutes, and will prove valuable for tissue typing and determining individual identity and disease susceptibility.

The present invention provide methods for detecting and identifying HLA DRB alleles. The number of distinct DRB1 alleles identified is continually increasing. The 1989 WHO Nomenclature Committee report listed 34 DRB1 alleles; this set of alleles is herein referred to as the 1989 allele set. The 1990 WHO Nomenclature Committee
5 report listed 45 alleles; this set of alleles is herein referred to as the 1990 allele set. In addition, the present invention provides the sequences of several newly discovered alleles.

The diversity of DRB loci, the most highly polymorphic of the HLA Class II loci, and the large number of alleles at these loci in the population make difficult the
10 process of identifying the particular DRB loci and alleles from which a nucleic acid in a sample originates. The present invention allows one to make this determination with great specificity and so can be used to identify the particular individual from whom a sample was taken. This discrimination power in turn leads to the applications of the invention in the field of forensic science.

15 Because PCR (or other amplification processes) can be used to amplify very small amounts of DNA (or degraded DNA), the present invention can be used to type HLA DRB DNA from unusual sources, such as a buccal swab, a single hair, and even DNA from preserved ancient specimens. With the latter samples, analysis of the alleles from prehistoric sources, e.g., early hominids, is possible. For purposes of the
20 present invention, "amplification" is defined by any process that increases the amount of target nucleic acid in a sample by means of nucleic acid replication or transcription.

The present method can be used for DRB DNA typing of cells that do not express the DRB genes. The method is also suitable, however, for DRB DNA typing of cDNA synthesized from DRB mRNA. The latter method facilitates the study of the
25 expression of HLA DRB in various cell lines or tissues and can be used to determine if there is an association between HLA DRB expression and susceptibility to transformation, autoimmunity, or other health conditions.

The research potential of the present invention should in no way obscure the immediate clinical applications. The genes and gene products of the MHC play a
30 central role in the immunological state of an individual, and particular MHC gene products are associated with disease resistance and susceptibility. Because the present invention allows the determination of the MHC DRB gene products in a sample, the invention also has applications in the field of medicine, particularly for medical diagnostic methods.

35 The discriminating power of this system will be valuable in typing potential transplantation donors, where very precise HLA DRB matching appears to be critical in minimizing risk of rejection or graft versus host disease. See the article entitled "Mixed lymphocyte reactions for individuals with phenotypic identity for specific HLA B, DR

determinants: The role of linkage disequilibrium and of specific DR and other Class II determinants" by Pollack *et al.*, 1983, *J. Clin. Immunol.* 3:341. Disease susceptibility studies have shown that single nucleotide differences in the DRB alleles can be medically significant. See the articles entitled "Specific HLA-DQB and HLA-DRB1 alleles confer susceptibility to *Pemphigus vulgaris*" by Scharf *et al.*, 1989, *Proc. Natl. Acad. Sci. USA* 86:6215; and "Identification of HLA-Dw14 genes in DR4+ Rheumatoid Arthritis" by Nepom *et al.*, 1986, *Lancet* page 1002.

In addition to the above benefits, the present invention also provides methods for identifying previously unknown DR alleles, and related primers, probes, and methods for the identification of any DRB allele. Unusual patterns of SSO probe hybridization using this typing system identify new alleles, as exemplified by the DNA typing of the "Beguit" cell line (see Example 7). This cell line showed an unusual pattern of probe hybridization at the DRB1 locus, revealing a previously unreported sequence, now designated DRB1*1103 (SEQ ID NO: 27).

In similar fashion, analysis by the present method of the DRB1 alleles in a patient with Lyme disease revealed a new pattern of probe hybridization. DRB1 allele sequences were cloned from genomic DNA of the patient and sequenced. The sequence of one DRB1 allele of the patient is different from any previously reported DRB1 allele and was originally designated as DR'LY10' (also DRB1*LY10). The other allele in the patient was DRB1*0402. The DR'LY10' allele has regions of sequences similar to DRB1*0801, *0802, *0803, and *1201 at the 5'-end and to *1401 at the 3'-end. The present invention provides probes for distinguishing the DR'LY10' allele from other DRB alleles. The DR'LY10' allele is now designated as DRB1*1404 (SEQ ID NO: 40).

Other newly discovered alleles of the DRB1 locus are DRB1*1305 (SEQ ID NO: 36), originally designated as DR'PEV', DRB1*1303 (SEQ ID NO: 34), and DRB1*1105 (SEQ ID NO: 29), originally designated as DRB1*BUGS. Each was discovered when analysis by the methods of the present invention revealed a new pattern of probe hybridization. Subsequent sequence analysis of each allele revealed the sequence variation causing the novel hybridization pattern. The present invention provides primers and probes for distinguishing the newly discovered alleles from other DRB alleles.

The present invention also provides kits for making practice of the present DRB typing method more convenient. One type of kit includes both amplification and typing reagents. Another kit contains only one or more DRB probes of the invention. In either kit, the probes can be labeled or unlabeled or attached to a solid support. The primers, if present in the kit, can also be labeled to facilitate detection, i.e., to bind a signal development reagent or for immobilization. The kits can also contain reagents

that facilitate detection of probe hybridization, i.e., the chromogenic substrate TMB and streptavidin-linked horseradish peroxidase. In brief, the reagents useful in practicing the present method can be packaged in any configuration that promotes utilization of the invention.

- 5 The nucleotide sequence of the second exon of each allele along with the encoded amino acid sequence are provided in the Sequence Listing section. Table 1 and 2, below, presents equivalent nucleotide sequence information for the alleles in the 1989 allele set, but in a manner which facilitates comparison. Table 1 also shows the nucleic acid sequence and the encoded amino acid sequence in both three and one letter codes for allele DRB*0101. Similarly, Table 3 presents equivalent amino acid sequence information, but using one letter amino acid codes. The sequence identification number of each allele is shown below. All alleles with the exception of DRB1*1503, DRB1*0303, and DRB1*1105 are listed in the 1990 WHO Nomenclature Committee report, *supra* (1990 allele set). For the sequence of
- 10 DRB1*1503, see also Demopolos *et al.*, 1991, *Human Immunology* 30:41-44.

15 The nucleotide sequences of alleles which do not appear in Tables 1, 2, and 3 are provided in Table 9 below in addition to the Sequence Listing section.

	<u>Allele</u>	<u>SEQ ID NO:</u>	<u>Allele</u>	<u>SEQ ID NO:</u>
20	DRB1*0101:	SEQ ID NO: 1	DRB1*1201:	SEQ ID NO: 30
	DRB1*0102:	SEQ ID NO: 2	DRB1*1202:	SEQ ID NO: 31
	DRB1*0103:	SEQ ID NO: 3	DRB1*1301:	SEQ ID NO: 32
	DRB1*0301:	SEQ ID NO: 4	DRB1*1302:	SEQ ID NO: 33
	DRB1*0302:	SEQ ID NO: 5	DRB1*1303:	SEQ ID NO: 34
25	DRB1*0303:	SEQ ID NO: 6	DRB1*1304:	SEQ ID NO: 35
	DRB1*0401:	SEQ ID NO: 7	DRB1*1305:	SEQ ID NO: 36
	DRB1*0402:	SEQ ID NO: 8	DRB1*1401:	SEQ ID NO: 37
	DRB1*0403:	SEQ ID NO: 9	DRB1*1402:	SEQ ID NO: 38
	DRB1*0404:	SEQ ID NO: 10	DRB1*1403:	SEQ ID NO: 39
30	DRB1*0405:	SEQ ID NO: 11	DRB1*1404:	SEQ ID NO: 40
	DRB1*0406:	SEQ ID NO: 12	DRB1*1405:	SEQ ID NO: 41
	DRB1*0407:	SEQ ID NO: 13	DRB1*1501:	SEQ ID NO: 42
	DRB1*0408:	SEQ ID NO: 14	DRB1*1502:	SEQ ID NO: 43
	DRB1*0409:	SEQ ID NO: 15	DRB1*1503:	SEQ ID NO: 44
35	DRB1*0410:	SEQ ID NO: 16	DRB1*1601:	SEQ ID NO: 45
	DRB1*0411:	SEQ ID NO: 17	DRB1*1602:	SEQ ID NO: 46
	DRB1*0701:	SEQ ID NO: 18	DRB2*0101:	SEQ ID NO: 47
	DRB1*0801:	SEQ ID NO: 19	DRB3*0101:	SEQ ID NO: 48

	DRB1*0802:	SEQ ID NO: 20	DRB3*0201:	SEQ ID NO: 49
	DRB1*0803:	SEQ ID NO: 21	DRB3*0202:	SEQ ID NO: 316
	DRB1*0804:	SEQ ID NO: 22	DRB3*0301:	SEQ ID NO: 50
	DRB1*0901:	SEQ ID NO: 23	DRB4*0101:	SEQ ID NO: 51
5	DRB1*1001:	SEQ ID NO: 24	DRB5*0101:	SEQ ID NO: 52
	DRB1*1101:	SEQ ID NO: 25	DRB5*0102:	SEQ ID NO: 53
	DRB1*1102:	SEQ ID NO: 26	DRB5*0201:	SEQ ID NO: 54
	DRB1*1103:	SEQ ID NO: 27	DRB5*0202:	SEQ ID NO: 55
	DRB1*1104:	SEQ ID NO: 28		
10	DRB1*1105:	SEQ ID NO: 29		

In Tables 1, 2, and 3, below, all sequences except the recently identified "PEV" and "LY10" alleles are listed in the 1989 WHO HLA nomenclature report, *supra*, and the DRB1*0101 nucleotide sequence serves as the consensus sequence. The inferred amino acid sequence for the consensus sequence is written in one and three letter code above the nucleotide sequence. Sequence homology is indicated by dashed lines, and letters indicate polymorphic bases. The designated SSO probes and primers written at the right end of the alignments in the tables are the same sense as, or complementary to, the regions of sequences boxed. Where two names appear at the end of an alignment, the left-most name refers to the left-most box, the right-most name refers to the right-most box. The probe CRX12 hybridizes to the region shown in all DRB alleles.

Table 1, in three parts designated A, B, and C, shows the nucleotide sequence of 35 DRB1 alleles corresponding to the DR specificities DR1 to DRw18.

Table 2 shows the nucleotide sequence alignments for the alleles of the DRB2, DRB3, DRB4, and DRB5 loci. The major regions of sequence polymorphism for the DRB1 alleles are localized to amino acid positions 9-16, 25-34, 67-74, and 86; the remainder of the second exon sequence is relatively invariant.

Table 3 shows the inferred amino acid sequence alignment encoded by the DRB alleles. Analysis of the amino acid sequences reveals a complex but restricted pattern of polymorphism with particular polymorphic sequences found in several different alleles. However, some polymorphic sequences are unique for each allele. The DR1, DR2, DR4, DR7, DRw9, and DRw10 alleles each have unique polymorphic sequences at the first hypervariable region (positions 9 to 16) of the DRB1 locus that can be used to determine the serological DR specificities by SSO typing. By contrast, the DR3, DRw11, and DRw6 alleles share a polymorphic epitope, "YSTS," and cannot be distinguished by a probe for this region alone but can be distinguished by the polymorphisms at other positions in each allele. Similarly, DRw8 and DRw12 also cannot be distinguished in this region.

Table 1A

LA - DR BETA DNA SEQUENCE ALIGNMENT									
	+10	+15	+20	+25	+30				
	R F L W Q L K F E C H F F N G T E R V R L L E R C I Y N Q								
B1*:	ArgPheLeuTrpGlnLeuLysPheGluCysHisPhePheAsnGlyThrGluArgValArgLeuGluArgCysIleTyrAsnGln								
101:	CACGTTTCTTGTGGCAGCTTAAGTTTCAATGTCATTCTTCAATGGACGGAGCGGGTGCCTGCTGGAAGATGCATCTATAACCAA					CRX60			
102:	-----								
103:	-----								
501:	-----C-----C-----AGG-G-----								GH104
502:	-----C-----C-----AGG-G-----								
601:	-----C-----C-----AGG-G-----								
602:	-----C-----C-----AGG-G-----								
1301:	-----GA-T-CTC-C-C-G-----								GH56
1302:	-----GA-T-CTC-C-C-G-----								
1401:	-----GA-G-----ACA-G-----								GH59
1402:	-----GA-G-----ACA-G-----								
1403:	-----GA-G-----ACA-G-----								
1404:	-----GA-G-----ACA-G-----								
1405:	-----GA-G-----ACA-G-----								
1406:	-----GA-G-----ACA-G-----								
1407:	-----GA-G-----ACA-G-----								
1408:	-----GA-G-----ACA-G-----								GH56
1101:	-----GA-T-CTC-C-C-G-----								
1102:	-----GA-T-CTC-C-C-G-----								
eguit:	-----GA-T-CTC-C-C-G-----								
1104:	-----GA-T-CTC-C-C-G-----								
1201:	-----GA-T-CTC-C-C-GG-G-T-----								GH102
1301:	-----GA-T-CTC-C-C-G-----								GH56
1302:	-----GA-T-CTC-C-C-G-----								
1303:	-----GA-T-CTC-C-C-G-----								
1401:	-----GA-T-CTC-C-C-G-----								
1402:	-----GA-T-CTC-C-C-G-----								
R'PEV':	-----GA-T-CTC-C-C-G-----								GH102
R'LY10:	-----GA-T-CTC-C-C-GG-G-T-----								CRX49
0701:	-----C-----GG-----A-A-G-----								GH102
0801:	-----GA-T-CTC-C-C-GG-G-T-----								
0802:	-----GA-T-CTC-C-C-GG-G-T-----								
0803:	-----GA-T-CTC-C-C-GG-G-T-----								
0901:	-----AA-----GA-----G-----								GH111
1001:	-----GA-G-G-----G-----								CRX34

Table 1B

	+35	+40	+45	+50	+55	+60	
DRB1*:	E E S V R F D S D V G E Y R A V T E L G R P D A E Y W N S Q						
0101:	GlutSerValIArgPheAspSerAspValGlyGluTyrArgAlaValThrGluLeuGlyArgProAspAlaGluTyrTrpAsnSerGln						CRX12
0102:	GAGGACTCGTGGCTTCGACACGACGCTGGGGGAGTACCGGGCGGTGACGGAGCTGGGGCGGCCCTGATGCCGAGTACTGGACAGCCAG						
0103:	---	---	---	---	---	C-T	
1501:	---	---	T	---	---	C-T	
1502:	---	---	T	---	---	C-T	
1601:	---	---	---	---	---	C-T	
1602:	---	---	---	---	---	C-T	
0301:	AA	---	T	---	---	---	
0302:	AA	---	---	---	---	---	
0401:	A	---	---	---	---	---	
0402:	A	---	---	---	---	---	
0403:	A	---	---	---	---	---	
0404:	A	---	---	---	---	AGC	CRX61
0405:	A	---	---	---	---	---	
0406:	---	---	---	---	---	---	
0407:	---	---	---	---	---	---	
0408:	A	---	---	---	---	AG	GH122
1101:	A	---	T	---	---	AG	
1102:	A	---	T	---	---	AG	
Beguit:	A	---	T	---	---	AG	
1104:	A	---	T	---	---	AG	
1201:	CT-C	---	T	---	---	TC	GH54
1301:	AA	---	T	---	---	---	
1302:	AA	---	T	---	---	AGC	CRX61
1303:	A	---	---	---	---	C-G-C	CRX23
1401:	T	---	---	---	---	---	
1402:	AA	---	---	---	---	---	
DR'PEV':	AA	---	T	---	---	C-G-C	CRX23
DR'LY10:	T	---	---	---	---	TC	GH54
0701:	T	---	---	---	---	AGC	CRX61
0801:	A	---	---	---	---	---	
0802:	A	---	---	---	---	AGC	CRX61
0803:	A-A	---	---	---	---	T	GH54
0901:	AA	---	---	---	---	---	
1001:	A-C	---	---	---	---	---	

Table 1C

	+65	+70	+75	+80	+85	+90	
DRB1*	K D L L E Q R R A A V D T Y C R H N Y G V G E S F T V Q R R						
0101:	LysAspLeuGluGlnArgAlaValAspThrTyrCysArgHisAsnTyrGlyValGlyGluSerPheThrValGlnArgArg						CRX04, CRX56
0102:	AGGACCTCTGAGCAGAGCGGGCG	AG-CGA					CRX06, CRX56
0103:		GC					CRX57
1501:							CRX56
1502:							CRX56
1601:							CRX35, CRX56
1602:							CRX50, CRX57
0301:							CRX56
0302:							CRX53
0401:							CRX06, CRX57
0402:							CRX15
0403:							CRX04
0404:							CRX56
0405:							CRX57
0406:							CRX56
0407:							CRX04
0408:							CRX35
1101:							CRX06, CRX57
1102:							CRX68
Beqult:							CRX35
1104:							CRX63
1201:							CRX06, CRX57
1301:							CRX56
1302:							CRX57
1303:							CRX56
1401:							CRX62
1402:							CRX04, CRX56
DR'PEV1:							CRX35
DR'LY10:							CRX56
0701:							CRX56
0801:							CRX63
0802:							CRX56
0803:							CRX56
0901:							CRX56
1001:							CRX56

Table 2

[illegible]

Table 3

ALIGNMENT OF ILA-DRB1 PROTEIN SEQUENCES									
	10	20	30	40	50	60	70	80	90
CONSENSUS	RFLEQ	K*ECHFTNGT	RVRF	LDYFY	QEEY	VRFDSDVGEY	RAVTELGRPD	AEYHNSOKDLEQ	*RAAVDTYCRHNYGVGESFTVQRR
DRB1*0101:	---	W-L-F	---	L-E-CI-N	---	S	---	R	---
DRB1*0102:	---	W-L-F	---	L-E-CI-N	---	S	---	R	---
DRB1*0103:	---	W-L-F	---	L-E-CI-N	---	S	---	I-DE	---
DRB1*1501:	---	W-P-R	---	N-S	---	F	---	I-A	---
DRB1*1502:	---	W-P-R	---	N-S	---	F	---	I-A	---
DRB1*1601:	---	W-P-R	---	N-S	---	F	---	F-DR	---
DRB1*1602:	---	W-P-R	---	N-S	---	F	---	F-DR	---
DRB1*0301:	---	YSTS	---	HN	---	N	---	K-GR	---
DRB1*0302:	---	YSTS	---	HN	---	N	---	K-GR	---
DRB1*0401:	---	V-H	---	H	---	H	---	K	---
DRB1*0402:	---	V-H	---	H	---	H	---	I-DE	---
DRB1*0403:	---	V-H	---	H	---	H	---	R-E	---
DRB1*0404:	---	V-H	---	H	---	H	---	R-E	---
DRB1*0405:	---	V-H	---	H	---	S	---	R-E	---
DRB1*0406:	---	V-H	---	H	---	S	---	R-E	---
DRB1*0407:	---	V-H	---	H	---	H	---	R-E	---
DRB1*0408:	---	V-H	---	H	---	H	---	R	---
DRB1*1101:	---	YSTS	---	N	---	F	---	F-DR	---
DRB1*1102:	---	YSTS	---	N	---	F	---	I-DE	---
DRB1*1103:	---	YSTS	---	N	---	F	---	F-DE	---
DRB1*1104:	---	YSTS	---	N	---	F	---	F-DR	---
DRB1*1301:	---	YSTS	---	HN	---	N	---	I-DE	---
DRB1*1302:	---	YSTS	---	HN	---	N	---	I-DE	---
DRB1*1303:	---	YSTS	---	N	---	S	---	I-DR	---
DRB1*1401:	---	YSTS	---	HN	---	F	---	RR-E	---
DRB1*1402:	---	YSTS	---	HN	---	N	---	R	---
"DR PEV"	---	YSTS	---	HN	---	N	---	F-DR	---
"LY10"	---	YSTG	---	Y	---	HN	---	A-H	---
DRB1*0701:	---	W-G-YK	---	Q-E-L-N	---	F	---	V-S	---
DRB1*0801:	---	YSTG	---	Y	---	N	---	S	---
DRB1*0802:	---	YSTG	---	Y	---	N	---	F-DR	---
DRB1*0803:	---	YSTG	---	Y	---	N	---	S	---
DRB1*0901:	---	K-D-F	---	Y-H-GI-N	---	N	---	V-S	---
DRB1*1001:	---	EV-F	---	L-E-RVIN	---	A-Y	---	RR	---
DRB1*1201:	---	YSTG	---	Y	---	L-E-H-HN	---	V-S	---
								I-DR	---
								AV	---
								DRW9	---
								DRW10	---
								DRW12	---

(BEGUIT)

Table 3 - Continued

	10	20	30	40	50	60	70	80	90																												
NSENSUS:	RFLEQ	*K	ECHFFNG	TERVR	FLDRYF	*Q	EYVR	FDSD	VGEYRA	VT	ELGR	PD	AEYWN	SQK	DL	LEQ	*R	AA	VD	TY	CR	HN	Y	G	V	G	E	S	F	T	V	Q	R				
B2*0101:	----	A	C	----	I	----	M	K	----	Q	Y	N	----	I	H	K	R	----	N	L	P	----	E	F	Q	----	V	N	----	G	I	----	E	N	D	K	----
B3*0101:	----	L	R	S	----	Y	----	H	N	----	F	L	----	V	S	----	K	G	R	N	----	DRW52a															
B3*0201:	----	L	L	S	----	E	H	H	N	----	A	----	R	----	K	G	Q	N	----	V	----	DRW52b															
B3*0301:	----	L	L	S	----	E	H	H	N	----	F	----	V	S	----	K	G	Q	N	----	V	----	DRW52c														
B4*0101:	----	A	C	----	L	----	W	N	I	----	I	N	----	A	Y	N	----	L	----	Q	----	Y	----	V	----												
B5*0101:	----	Q	D	Y	----	H	D	I	N	----	D	L	----	F	----	D	R	----	DR2-DW2																		
B5*0102:	----	Q	D	Y	----	H	G	I	N	----	N	----	F	----	D	R	----	DR2-DW12																			
B5*0201:	----	C	Q	D	Y	----	H	G	I	N	----	N	----	I	----	A	----	AV	----	DR2-DW21																	

The classical serologically defined DR types (except DR3 and DRw6) can be distinguished by amplifying with the generic DRB primers GH46 (SEQ ID NO: 67) and GH50 (SEQ ID NO: 68) and analyzing the amplification products with the first panel of probes, shown in Table 4. The allele specificities shown in Table 4 are with respect to the 1989 allele set. For purposes of the present invention, "generic primers" are PCR primers that hybridize to DRB gene second exon sequences and can be used to amplify any allele of any DRB locus. Because of the hybridization site of GH50, one cannot probe for polymorphisms at position 86 (referring to the amino acid sequence) of the second exon when the product of amplification was generated with primer GH50. An alternate primer that can be used to generate products than can be analyzed for position 86 polymorphisms is DRB151 (SEQ ID NO: 227), 5'-CCGAATTCGCCGCTGCACTGTGAAGCT-3'.

Table 4
First Panel of HLA DRB Typing SSO Probes

15	HLA DRB				
	Probe	SEQ ID NO:	Epitope	Alleles (DRB1*)	Wash (SSPE, °C.)
	CRX60	SEQ ID NO: 79	"W-L-F"	0101, 0102, 0103	0.4X, 42
	GH105	SEQ ID NO: 91	"Q-D-Y"	DRB5	0.1X, 42
	GH104	SEQ ID NO: 90	"W-P-R"	1501, 1502, 1601, 1602	0.2X, 42
20	GH59	SEQ ID NO: 87	"V-H"	0401-0408	0.2X, 42, 20'
	CRX06	SEQ ID NO: 61	"I-DE"	0103, 0402, 1102, 1301, 1302	0.1X, 42
	GH122	SEQ ID NO: 93	"E"	1101, 1102, 1103, 1104	0.2X, 42
	CRX23	SEQ ID NO: 66	"A-H"	1401, DR "LY10"	0.1X, 42
25	CRX35	SEQ ID NO: 71	"F-DR"	1601, 1101, 1104, DR "PEV", 0801, 0802	0.2X, 42
	CRX49	SEQ ID NO: 74	"G-YK"	0701, 0702	1.0X, 42
	GH102	SEQ ID NO: 89	"YSTG"	0801, 0802, 0803, 1201, DR "LY10", 1404	0.1X, 42
30	GH111	SEQ ID NO: 92	"K-D-F"	0901	0.4X, 42
	CRX34	SEQ ID NO: 70	"EV"	1001	0.4X, 42
	CRX04	SEQ ID NO: 60	"R"	0101, 0102, 0403, 0404, 0405, 0406, 0407, 0408, 1402	0.1X, 42
35	GH56	SEQ ID NO: 86	"YSTS"	0301, 0302, 1101-1104, 1301-1303, 1401, 1402, DR "PEV"	0.2X, 42
	CRX68	SEQ ID NO: 84	"F-DE"	1103	0.2X, 42
	CRX12	SEQ ID NO: 63	DRB "ALL"	All HLA-DRB alleles	0.2X, 42

The probes shown in Table 4 are hybridized and then washed for 15 minutes at 42°C, except for GH59, which is washed for 20 minutes at 42°C. All SSPE wash solutions contain 0.1% SDS. Each probe is conjugated to HRP at the 5'-end.

This PCR/SSO DRbeta typing system is useful for "subtyping" the serologically defined DR haplotypes. For example, the cell line "KOSE" is homozygous for DRw6 when typed serologically; however, PCR/SSO DRbeta typing reveals that "KOSE" has two different DRw6 alleles, DRB1*1302 and DRB1*1401.

Samples that may be DR3 or DRw6 can be distinguished by amplifying with the DRB1 specific primers GH46 and CRX37 and analyzing the amplification products with the probes GH125 and CRX50 from the second panel of probes, shown in Table 5. The allele specificities shown in Table 5 are with respect to the 1989 allele set.

Table 5
Second Panel of HLA DRB Typing SSO Probes

				HLA DRB	
15	Probe	SEQ ID NO:	Epitope	Alleles (DRB1*)	Wash (SSPE, °C.)
	GH125 ^a	SEQ ID NO: 94	"Y"	0301	0.2X, 50
	CRX50 ^a	SEQ ID NO: 75	"K-GR"	0301, 0302	0.2X, 50
	CRX53 ^a	SEQ ID NO: 76	"K"	0401	0.5X, 55
	CRX15 ^a	SEQ ID NO: 64	"R-E"	0403, 0406, 0407	0.4X, 55
20	CRX62	SEQ ID NO: 81	"I-DK"	1303	0.2X, 42
	CRX63	SEQ ID NO: 82	"I-DR"	0803, 1201	0.2X, 42
	CRX61	SEQ ID NO: 80	"S"	0405, 1303, 0801, 0803	0.1X, 42
	GH54 ^a	SEQ ID NO: 85	"V-S"	0701, 0901, 1201	0.2X, 42
25	CRX56 ^a	SEQ ID NO: 86	"G"	0101, 0103, 0302, 0401, 0405, 0407, 0408, 1101, 1302, 1303, 0701, 0901, 1001, 1402, DR "PEV", 0801, 0802, 0803, 1502, 1601, 1602	0.2X, 42
30	CRX57 ^a	SEQ ID NO: 78	"V"	0301, 0402, 0403, 0404, 0406, 1102, 1103, 1104, 1301, DR "LY10," 1401, 1501	0.1X, 42
35	CRX12	SEQ ID NO: 63	DRB "ALL"	All HLA DRB alleles	0.2X, 42

For the probes shown in Table 5, all SSPE wash solutions contain 0.1% SDS. In addition, probes marked "a" require DRB1 specific amplification with primers GH46/CRX37. CRX15 is hybridized at 50°C instead of 42°C. Each probe is conjugated to HRP at the 5'-end. The probes CRX56 and CRX57 do not completely base-pair with the epitope "G" and epitope "V" alleles, respectively, because the probes

contain one fewer G residue than the alleles. Probes modified to include the G residue missing in CRX56 and CRX57 are within the scope of the present invention.

For purposes of the present invention, "DRB1 specific" primers are primer pairs that comprise at least one primer that hybridizes to an intron sequence flanking the second exon of the DRB1 gene, which primer will not hybridize to the other DRB genes. Table 6 shows the nucleotide sequence of the DRB1 and DRB3 introns of the DR3 haplotype, from the last 10 codons prior to the start of the 3' downstream intron. The sequence for CRX37 (SEQ ID NO: 73) is underlined; the solid arrow indicates the direction of extension of the primer. The asterisks indicate sequence differences between the DRB1 and DRB3 intron sequences. The segments of the 3' downstream introns are listed in the Sequence Listing as SEQ ID NO: 314 (DRB1) and SEQ ID NO: 315 (DRB3); sequences upstream of the intron are in the allele sequences.

Table 6

		85	90	
15	AA Seq:	Val Val Glu Ser Phe Thr Val Gln Arg Arg	←	<u>TCGCGCGCGCGCCCTTAAG</u> CRX37
	DRB1 NT:	GTT GTG GAG AGC TTC ACA GTG CAG CGG CGA	GGTGAGCGCGCGCGCGGGG.....	
				* * * * *
	DRB3 NT:	GTT GTG GAG AGC TTG ACA GTG CAG CGG CGA	GGTGAGCATGTCGGGGGGCGG.....	
	<u>Intron</u>	<u>Seq. ID No.</u>	<u>Sequence</u>	
20	DRB1	SEQ ID NO: 314	5'-GGTGAGCGCGCGCGCGGGG	
	DRB3	SEQ ID NO: 315	5'-GGTGAGCATGTCGGGGGGCGG	

The DR3, DR4, and DRw6 haplotypes appear to be evolutionarily distinct from DR2, DR7, and DR9 haplotypes. There may be differences in the intron sequences of these alleles such that the primer is too mismatched to prime extension. Because the DRB1 alleles may have diverged in evolution prior to gene duplication, it may prove difficult to find DRB1 specific primer sequences for amplifying the DRB1 gene second exon from all DR haplotypes.

The HLA DRB1 alleles can be distinguished with greater specificity by amplifying with the generic and DRB1 specific primers and analyzing with both panels (Tables 4 and 5) of probes as required. The alleles of the 1989 allele set that cannot be distinguished with the specific probes in Tables 4 and 5 are the four DR2 subtypes DRB1*1501, *1502, *1601, and *1602 and the DR4 subtypes DRB1*0403 and DRB1*0406.

The DRB1 specific PCR primers are capable of reproducibly amplifying the DRB1 sequences from all but the DR2, DR7, and DR9 haplotypes. The primer CRX37 was designed from intron nucleotide sequences from DR3, DR4, and DRw6 haplotypes. See the article entitled "Sequence Analysis of HLA Class II Genes from Insulin-Dependent Diabetic Individuals" by Horn *et al.*, 1988, *Hum. Immunol.* 1:249.

The present invention provides a number of allele specific and group specific primers in addition to the DRB1 specific primer pair GH46/CRX37. These primers are shown below. For the sequence of AB60, see Todd *et al.*, 1987, *Nature* 329:599.

	AB54	SEQ ID NO: 56	5'-GGGGATCCTGGAGCAGGTAAACA-3'
5	AB60	SEQ ID NO: 57	5'-CCGAATTCCGCTGCACTGTGAAGCTCTC-3'
	AB82	SEQ ID NO: 58	5'-GGGGATCCTGGAGTACTCTACGTC-3'
	AB83	SEQ ID NO: 59	5'-GGGGATCCTGTGGCAGCCTAAGAGG-3'

For example, DR4 specific amplification can be achieved with PCR primer pair AB54/AB60 (PCR profile: 35 cycles of: ramp to 94°C; 30 seconds denaturing at 94°C; 10 30 seconds annealing and extending at 65°C). DR3, DR5, and DR6 group specific amplification can be achieved with PCR primer pair AB82/AB60 (35 cycles of: ramp to 96°C; 30 seconds denaturing at 96°C; 30 seconds annealing and extending at 65°C). DR2 specific amplification can be achieved with PCR primer pair AB83/AB60 (35 cycles of: ramp to 96°C; 30 seconds denaturing at 96°C; 30 seconds annealing and 15 extending at 70°C).

Specific alleles can be detected after group specific amplification with the epitope specific probes described herein; for instance, DRB1*1601 can be detected after DR2 specific amplification with the AB83/AB60 primer pair with an "F-DR" epitope specific probe.

20 Detection of alleles of the DR2 haplotype can be difficult, but such difficulty can be overcome by the group specific primers and the typing methods of the present invention. In the generic primer amplification of DRB alleles from DR2 haplotypes, both the DRB1 and DRB5 loci are amplified. Although one could use an "F" (at position 47) epitope specific DNA probe to determine the serologic DRw15 and 25 DRw16 subtypes, "F" occurs also in 0301, some DRw13 alleles, and in all DRw11 and DRw12 alleles; other methods can provide more complete discrimination. Thus, a group specific primer designed to hybridize to the sequence encoding the epitope "I--A" will amplify the DRB1*1501 and DRB1*1502 alleles, and the amplified product will hybridize with a "WPR" epitope specific DNA probe. The primer DRB150 (SEQ ID 30 NO: 226) (5'-TGTCCACCGCGGCCCCGCGCCT-3') is a primer designed to perform such an allele specific amplification (with GH46). The DRB5*0201 and DRB5*0202 alleles will also amplify with a group (or epitope) specific "I--A" primer, but as these alleles occur only with the DRB*1601 and DRB1*1602 alleles, the result of probe hybridization will be epitope "QDY" positive and "WPR" negative.

35 In addition, one should note that because the DRB1 specific primers exemplified herein do not amplify DR2 alleles, the DR2 specific amplification described above can be carried out in the same reaction tube and at the same time as the DRB1

specific amplification. Other allele and group specific primers and amplification methods are described in the Examples.

The extensive allelic diversity at the HLA DRB loci, like that of the other Class II beta genes, is localized primarily to the second exon. In general, the pattern of second exon sequence polymorphism, when present in the population at a particular DRB locus, is a patchwork, with specific polymorphic segments found in a variety of different alleles. In principle, such shared epitopes among different alleles could reflect either common ancestry, gene conversion, or convergent evolution. For purposes of oligonucleotide typing, however, this patchwork pattern of polymorphism means that many alleles cannot be identified by hybridization to a single oligonucleotide but can be identified by a unique pattern of hybridization with a panel of probes.

The sequence-specific oligonucleotide probes of the invention, called "SSOs," when employed in the present methods under the appropriate hybridization and wash conditions, are extremely specific, capable of distinguishing single nucleotide polymorphisms. The SSOs of the invention, unlike the probes used in RFLP methods, can be used to determine not only if alleles are different but also where and how the alleles differ.

In a preferred embodiment, these probes comprise the same sequence as, or a sequence complementary to, the regions of DNA of each allele boxed in Tables 1 and 2. For example, the probe CRX60 is specific for the DNA encoding the amino acids "W-L-F" and can hybridize uniquely to the DR1 allele. The probe CRX49 is specific for the amino acids encoding "G-YK" and hybridizes to the DR7 allele; other examples will be apparent upon consideration of the Tables.

While a single probe (i.e., GH56) can be used to distinguish DR3, DRw11, and DRw6 from the other DR serotypes, additional probes are required to distinguish between DR3, DRw11, and DRw6. The GH56 + GH122 probe combination, which hybridizes to DNA encoding the "YSTS" and "E" epitopes (codons 10 to 13 and 58) on DRw11, can be used to distinguish DRw11 from DR3 and DRw6. Likewise, the probe combinations GH56 + CRX06 corresponding to the "YSTS" and "I-DE" (codon 67 to 71) epitopes can be used to distinguish DRw6 (DRB1*1301 and DRB1*1302 but not DRB1*1303, which has the epitope "I-DK" but is still DRw6) from DRw11 and DR3.

The combinations of the SSO probes shown in Table 4 used to determine the majority of the serological DR types are shown in Table 7.

Table 7Combinations of SSO Probes to Determine Serological DR Types

	<u>DR Type</u>	<u>Probe(s)</u>
5	DR1 Dw1	CRX60 + CRX04
	DR1 Dw "BON"	CRX60 + CRX06
	DR2	GH105
	DR4 Dw4	GH59 + CRX53
	DR4 Dw10	GH59 + CRX06
10	DR4 Dw13, 14, 15	GH59 + CRX04
	DR3, w11, w6	GH56
	DRw11.1	GH56 + GH122 + CRX35
	DRw11.2	GH56 + GH122 + CRX06
	DRw12, DRw8.3	GH102 + CRX63
15	DRw13 (1301, 1302)	GH56 + CRX06
	DRw14 Dw16	GH56 + CRX04
	DRw14 Dw9	GH56 + CRX23
	DR7	CRX49
	DRw8.1, w 8.2	GH102 + CRX35
20	DR9	GH111
	DRw10	CRX34
	DRw12, all DRw8	GH102

Further subdivision of the DR serotypes (Table 4) requires the use of probes shown in Table 5, as shown in Table 8, below.

Table 8

Combinations of SSO Probes to Distinguish 31 of 34 HLA DRB1 Alleles (1989 set)

DRB1*	Probe(s)	DRB1*	Probe(s)
5	0101 CRX60 + CRX04 + CRX56	1103	GH122 + GH56 + CRX57 + CRX68
	0102 CRX60 + CRX04	1104	GH122 + GH56 + CRX35 + CRX57
	0103 CRX60 + CRX06 + CRX56	1201	GH102 + GH54 + CRX63
	DR2 GH105	1301	GH56 + CRX06 + CRX57
	0301 GH56 + GH125 + CRX50 + CRX57	1302	GH56 + CRX06 + CRX56
10	0302 GH56 + CRX50 + CRX56	1303	GH56 + CRX62 + CRX61 + CRX56
	0401 GH59 + CRX53 + CRX56	1401	GH56 + CRX23 + CRX57
	0402 GH59 + CRX06 + CRX57	1402	GH56 + CRX04 + CRX56
	0403, 0406 GH59 + CRX15 + CRX57 + CRX04	DR"PEV"	GH56 + CRX35 + CRX56
	0404 GH59 + CRX04 + CRX57	0701	CRX49 + GH54 + CRX56
15	0405 GH59 + CRX04 + CRX61 + CRX56	0801	GH102 + CRX61 + CRX35 + CRX56
	0407 GH59 + CRX15 + CRX56 + CRX04	0802	GH102 + CRX35 + CRX56
	0408 GH59 + CRX04 + CRX56	0803	GH102 + CRX61 + CRX63 + CRX56
	1101 GH122 + GH56 + CRX35 + CRX56	0901	GH111 + GH54 + CRX56
	20 1102 GH122 + GH56 + CRX06 + CRX57	1001	CRX34 + CRX56
			DR"LY10" GH102 + CRX23 + CRX57

The horseradish peroxidase-conjugated SSOs of the invention, called "HRP-SSOs," allow detection methods that employ chromogenic or chemiluminescent substrates that are easy to use and produce detectable signals rapidly (typically 1 to 10 minutes). The HRP-SSOs are stable for over two years without detectable loss of activity when stored at 4°C. See the article entitled "Nonisotopically labeled probes and primers" by Levenson and Chang, 1989, in PCR Protocols: A Guide to Methods and Applications (Innis, Gelfand, Sninsky and White ed., Academic Press, Inc. San Diego). Radiolabelled probes can be employed but are not necessary for excellent sensitivity, an important benefit provided by the present invention.

The dot-blot format for detection enables the rapid typing of a large number of samples and will be useful in determining the allele frequencies of HLA DRB. A recently developed alternative for PCR/SSO DRbeta typing is the immobilized reverse dot-blot format. See the article entitled "Genetic analysis of amplified DNA with immobilized sequence-specific oligonucleotide probes" by Saiki et al., 1989, Proc. Natl. Acad. Sci. USA 86:6230, and copending Serial No. 347,495, filed May 4, 1989, incorporated herein by reference. In this procedure, the SSO probes are applied and

fixed to the filter (rather than the amplified DNA applied and fixed to the filter), hence the term "reverse dot blot."

The reverse dot blot procedure allows a single sample to be analyzed in a single hybridization with a membrane containing an array of immobilized probes. The conventional dot blot format is useful when the number of samples exceeds the number of probes used (e.g., patient versus control or population genetics studies). The reverse dot blot format is valuable for clinical, diagnostic, and forensic analyses. The reverse dot blot format is described in more detail in Example 8.

The following examples show illustrative preferred embodiments of the present invention. The examples show that the present invention provides, in a preferred embodiment, a nonisotopic PCR/SSO system for HLA DRB typing that is simple, rapid, and capable of precise DRB typing for a variety of samples from different sources.

Example 1

15

Amplification and Detection Methods

For typing samples with the first panel (Table 4) of probes, 0.5 µg of human genomic DNA was amplified using reaction constituents as described in the article entitled "Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase," by Saiki *et al.*, 1988, *Science* 239:487 and in Scharf *et al.*, 1988, *Hum. Immunol.* 22:61, and Scharf *et al.*, 1989, *Proc. Natl. Acad. Sci. USA* 86:6215, incorporated herein by reference.

The HLA DRB generic PCR primers are GH46 and GH50. The sequences of these primers are shown below.

GH46 SEQ ID NO: 67 5'-CCGGATCCTTCGTGTCCCCACAGCACG
25 GH50 SEQ ID NO: 68 5'-CTCCCCAACCCCGTAGTTGTGTCTGCA

The primers were present in the reaction mixture at 500 nM. These primers produce a 272 base-pair (bp) fragment and contain sequences for BamHI and PstI restriction sites for cloning the PCR product. Digestion of the amplified DNA with BamHI and PstI produces a 248 bp product due to an internal PstI site.

30 The DRB1 alleles from all haplotypes (except DR2, DR7, and DR9) were specifically amplified by the PCR primers GH46 and CRX37. The sequence of the CRX37 primer is shown below.

CRX37 SEQ ID NO: 73 5'-GAATTCCCGCGCCGCGCT

Amplification with the GH46/CRX37 primer pair produces a 297 bp fragment. Primer
35 CRX37 incorporates an EcoRI restriction endonuclease recognition sequence at the 5'-end to facilitate cloning and, in contrast to the primer pair GH46/GH50, amplification

and BamHI/EcoRI digestion with this primer pair allows isolation and analysis of the full-length PCR product.

Such isolation and analysis frequently involved the determination of the nucleotide sequence of the PCR product. For sequencing the HLA DRB alleles, 1 µg of purified human genomic DNA was amplified using the primer pairs GH46/GH50 and GH46/CRX37. The amplified DNA was cloned into M13mp10 by the methods described in Scharf *et al.*, 1988, Hum. Immunol. 22:61, and Scharf *et al.*, 1986, Hum. Immunol. 233:1076, incorporated herein by reference. The inserts were then sequenced by the dideoxy chain-termination procedure described in Sanger *et al.*, 1977, Proc. Natl. Acad. Sci. USA. 74:5463 (see also U.S. patent application Serial No. 249,367, filed September 23, 1988, and incorporated herein by reference).

The sequences shown in Tables 1 and 2 above were generated by genomic cloning (see Horn *et al.*, 1988, Hum. Immunol. 21: 249, incorporated herein by reference), PCR amplification (see Erlich *et al.*, 1989, in Immunobiology of HLA (du Pont ed., Springer-Verlag, New York) and Scharf *et al.*, 1989, Proc. Natl. Acad. Sci. USA 86: 6215, incorporated herein by reference), or from the literature (see WHO Nomenclature Committee, 1990, Immunogenetics 31:131 and Gregersen *et al.*, 1989, "First domain diversity of DR and DQ subregion alleles" in Immunobiology of HLA (du Pont, ed., Springer-Verlag, New York)).

Samples were amplified for 32 cycles (unless otherwise noted) using the reaction conditions described above, except that 1.25 units (rather than 2.5 units) of Taq polymerase (PECI, Norwalk, CT) were added per 100 µl of reaction volume. The cDNAs for the bladder carcinoma patients were amplified for the HLA-DRB loci as described (see Kawasaki, 1989, "Amplification of RNA" in PCR Protocols: A Guide to Methods and Applications (Innis *et al.*, eds., Academic Press, San Diego)). All samples were overlaid with 100 µl of high grade mineral oil (Sigma, St. Louis, MO) to prevent evaporation. After amplification, the oil overlay was extracted with 100 µl chloroform.

The thermal profile for each cycle comprised incubations at the following temperatures for the indicated times: 45 seconds at 94°C (for denaturation of the DNA strands), 45 seconds at 55°C (for annealing of the primers), and 45 seconds at 72°C (for extension of the primed templates). After the last cycle, the PECI Thermal Cycler (Perkin Elmer Cetus Instruments, Norwalk, CT) was programmed to incubate the samples at 72°C for 10 minutes to ensure that the final extension was complete. Care should be taken to avoid cross-contamination of samples; one must particularly guard against allowing the product of one PCR to contaminate an unamplified sample. U.S. patent application Serial No. 557,517, filed July 24, 1990, and the CIP of that

application filed November 2, 1990, both incorporated herein by reference, describe preferred methods to prevent amplification of PCR product that has been "carried over" to an unamplified sample.

After amplification, a small portion of the amplified DNA was denatured and
5 applied to and crosslinked to a series of nylon filters; each filter was then hybridized to one of the labelled probes. Each SSO probe was covalently conjugated to horseradish peroxidase (HRP) and provides a means of nonisotopic detection in the presence of a chromogenic or chemiluminescent substrate. The nucleotide sequence, the encoded amino acids (or potential epitope), and the identified DR type, as well as the wash
10 conditions for each probe are listed in Tables 4 and 5.

Thus, 5 μ l of each amplified DNA sample were mixed with 100 μ l of a mixture composed of 0.4 M NaOH and 25 mM EDTA, and the resulting mixture applied to BioDyne B nylon filters (Pall Corp., Glen Cove, NY) using a dot-blot manifold (Bio Rad, Richmond, CA). The filters, still in the dot-blot manifold, were rinsed with a
15 mixture of 10 mM Tris-HCl and 0.1 mM EDTA, at pH 8.0, and dried on Whatman 3MM paper. The DNA was immobilized on the nylon filter by ultraviolet irradiation at a flux of 55 mJ/cm² with a Stratalinker™ (Stratagene, La Jolla, CA) UV light box.

Unless otherwise noted, all filters were hybridized in 2X SSPE (saline sodium phosphate EDTA), 5X Denhardt's solution, and 0.5% SDS with 2 pmoles of HRP-
20 SSO probe per ml of hybridization solution for 15 min. at 42°C. Horseradish peroxidase conjugated oligonucleotides were prepared as described by Levenson and Chang, 1989, in PCR Protocols: A Guide to Methods and Applications (Innis *et al.*, eds., Academic Press, Inc. San Diego) and Saiki *et al.*, 1988, N. Eng. J. Med. 319:537. Filters for each probe were washed in 25 ml of the SSPE solutions at the
25 temperatures listed in Tables 4 and 5 for 15 minutes (unless noted otherwise).

After washing, filters to be developed with a chromogenic dye substrate were rinsed in PBS at room temperature for 30 minutes, then placed in 100 mM sodium citrate, pH 5.0, containing 0.1 mg/ml of 3,3',5,5'-tetramethylbenzidine (TMB) per
30 milliliter (Fluka) and 0.0015 percent hydrogen peroxide, and incubated with gentle agitation for 5 to 15 minutes at room temperature. Developed filters were rinsed in PBS and immediately photographed. Filters that were developed with the chemiluminescent detection system (ECL; Amersham, Arlington Heights, IL) were rinsed in PBS for 5 minutes and placed in the ECL solution for 1 minute with gentle agitation. Filters were then exposed to X-ray film at room temperature for 1 to 5
35 minutes.

Example 2DRB1 Specific Amplification

Several polymorphic sequences of alleles of the DRB1 locus that distinguish the various DRB1 alleles are also present on alleles of the other DRB loci (see Table III).

5 An example is the nucleotide sequence encoding the epitope "K-GR" (codons 71 to 74) on DR3 DRB1 alleles, where a probe to this region (CRX50) can distinguish DR3 from DRw11 and DRw6 alleles. However, this epitope is also encoded by the DRB3 allele DRw52a (DRB3*0101) and is present on some DRw6 haplotypes and some DR3 haplotypes as well. Because the PCR primers GH46/GH50 amplify all of the DRB loci, a DRw6 sample that was DRw52a at the DRB3 locus would be impossible to
10 distinguish from a DR3 sample using this probe.

The present invention solves this problem by providing PCR primers that specifically amplify only the DRB1 locus. One of these primers hybridizes to a region in the intron immediately downstream from the second exon. The intron contains
15 sequences that distinguish the DRB1 and DRB3 loci. The primer (CRX37) hybridizes specifically to the DRB1 intron sequences, and combining this primer with GH46 permits DRB1 specific amplification for most haplotypes.

To determine if the DRB1 locus is the only locus amplified with these putative DRB1 specific primers, DR2, DR3, and DR4 HTC (homozygous typing cells) DNA
20 was amplified and analyzed with these primers and SSO probes for all the DRB loci. In addition to the DRB1 locus, the DR2 haplotype has a DRB2 and DRB5 locus, the DR3 haplotype has a DRB3 locus, and the DR4 haplotype has a DRB4 locus. The results are shown in Figure 1.

To generate these results, about 200 ng of HTC DNA were amplified by the
25 generic DRB primers GH46/GH50 or by the DRB1 specific primers GH46/CRX37 and applied to filters as described in Example 1. Each filter containing amplified cell line DNA (samples 1 to 4) was hybridized with the probes shown in Figure 1. The locus to which each probe hybridizes is shown in parentheses. CRX36 hybridizes to the DRB4 locus of DR4, DR7, and DR9. CRX22 hybridizes to the DRB3 allele
30 DRw52b.

The sequence of the SSOs:

CRX36 SEQ ID NO: 72 5'-CCCGCCTCCGCTCCA

CRX22 SEQ ID NO: 65 5'-GAGCTGCTTAAGTCT

is described by Scharf *et al.*, 1989, *Proc. Natl. Acad. Sci. USA* 86:6215, incorporated
35 herein by reference. Probe GH91 (SEQ ID NO: 88, 5'-CTCCCGTTTATGGATGTAT) hybridizes specifically to the DRB2 locus. This probe was hybridized as described in Example 1 and washed in 1X SSPE, 0.1% SDS

for 15 minutes at 42°C. The samples amplified are: (1) No DNA (negative control); (2) DR2 HTC "SCHU"; (3) DR3 HTC "QBL"; and (4) DR4 HTC "BSM." After the hybridization and wash steps, the probes were detected by enhanced chemiluminescence.

5 Figure 1 shows that all of the DRB loci for all three HTCs are amplified with the generic DRB primers, while the DRB1 specific primers only amplify the DRB1 loci from the DR3 and DR4 HTCs. Interestingly, for the DR2 HTC, the DRB2 locus is amplified, albeit weakly, with the DRB1 specific primers. The DRB1 specific primers amplify DRB1 sequences efficiently from all DR haplotypes tested except DR2, DR7,
10 and DR9, as shown in Figures 1 and 2. To generate the results shown in Figure 2, about 0.5 µg of genomic DNA from HTCs was amplified for HLA DRB with the DRB1 specific primers GH46/CRX37. Amplification reactions and filters were prepared as described in Example 1. Each filter strip was hybridized to one of the probes shown under the appropriate conditions of hybridization and wash stringency
15 (see Tables 4, 5, and 7). After the hybridization and wash steps, filters were developed in the chromogenic substrate TMB. The samples are: (1) DR1 HTC "KAS9003"; (2) DR2 HTC "SCHU"; (3) DR3 HTC "QBL"; (4) DR4 HTC "BSM"; (5) DR5 (DRw11) HTC "SPOO1O"; (6) DRw6 HTC "OMW"; (7) DR7 HTC "MOU"; (8) DRw8 HTC "SPACH"; (9) DR9 HTC "DKB"; and (10) DRw10 HTC "SHY".

20 The results in Figure 2 indicate that the DR7 probe CRX49 ("G-YK") does hybridize to the weakly amplified DR7 HTC in this experiment. DRB1 amplification of DR7 from heterozygous samples is typically weak. These results demonstrate that the generic and DRB1 specific primers can be used for complete DRB1 typing.

Example 3

Typing for the Serological DR Types 1 Through 10

25 About 0.5 µg of genomic DNA from HTCs was amplified for HLA DRB. All samples except for nos. 3 and 6 were amplified with the generic primers GH46/GH50. Sample nos. 3 and 6 were amplified with the DRB1 specific primers GH46/CRX37. Amplification reactions and filters were prepared as described in Example 1. Each filter
30 strip was hybridized to one of the probes shown under the appropriate conditions of hybridization and wash stringency (see Tables 4, 5, and 7). After probing and washing, filters were developed in the chromogenic substrate TMB. The samples are: (1) DR1 HTC "KAS9003"; (2) DR2 HTC "SCHU"; (3) DR3 HTC "QBL"; (4) DR4 HTC "BSM"; (5) DR5 (DRw11) HTC "SPOO1O"; (6) DRw6 HTC "OMW"; (7) DR7
35 HTC "MOU"; (8) DRw8 HTC "SPACH"; (9) DR9 HTC "DKB"; and (10) DRw10 HTC "SHY".

Figure 3 demonstrates the results of DR typing for the classical, serologically defined DR types 1 through 10 on a panel of HTCs. A single probe can be used to detect each of the classical DR types, but two different PCR primer pairs were required. For all but the DR3 and DRw6 samples, the generic PCR primers GH46/GH50 can be used. The DRB1 specific primer pair GH46/CRX37 was used for amplifying the DNA of the DR3 and DRw6 samples in this array, because the probe used to detect the "Y" epitope (GH125; codon 26) on the DR3 HTC "QBL" (DRw17) is also present on the DRB3 allele (DRw52a) of the DRw6 sample. If the standard DRB primers had been used, then the "Y" probe would have hybridized to the DRw6 sample as well.

10

Example 4

General DRB1 Typing Strategy

For typing a particular sample, the generic primers should be used for the first amplification, followed by probing of the amplified DNA with the first panel of probes (Tables 4 and 7). This panel of probes will identify the majority of the serological types; although GH56 ("YSTS") will not unequivocally distinguish DR3 from DRw6.

For samples that are positive with GH56, or for subtyping particular DR types (e.g., the Dw types of DR4 or the subtypes of DRw8), the second panel (Table 5) of probes should be used. As discussed above, some of the probes in the second panel require amplification with the DRB1 specific pair of PCR primers. By combining the results of the probings from the first and second panels, one can distinguish 31 of the 34 DRB1 alleles in the 1989 allele set. The combinations of SSO probes used to identify these alleles is shown in Table 8. The DR2 subtypes (DRB1*1501, *1502, *1601, and *1602) are not distinguished by these primers and probes, and the DRB1*0403 and DRB1*0406 alleles are not distinguished by these probes.

One DR2 probe of the invention comprises sequences that hybridize to the first hypervariable region ("Q-D-Y") of the DRB5 locus. Because the DRB5 locus is present on all known DR2 haplotypes and only on those haplotypes, such a probe can be used reliably to type for DR2 in an amplification with generic DRB primers. Probe GH104 ("W-P-R") hybridizes to the first hypervariable region of the DR2 DRB1 locus and hybridizes specifically to DR2 DNA when amplified with the generic primers (Figure 1) but not with the DRB1 specific primers. These two probes are identical indicators of DR2 in an amplification with generic primers.

Example 5Subtyping DR4

The subtyping of the DR4 specificity cannot be carried out serologically. The subtypes have been revealed by two dimensional protein gel electrophoresis and cellular
5 typing; both methods are cumbersome and time-consuming. Dw4, Dw10, Dw13, and Dw14 differ from each other at positions 70 to 74 of the third hypervariable region of the gene. Dw15 also has a serine ("S") at position 57. Dw13 also has the same arginine residue at position 71 as Dw14 and Dw15 but is distinguished by a glutamic acid ("E") as position 74.

10 As a result, the SSO probe CRX04 ("R") probe hybridizes to the Dw13, Dw14, and Dw15 alleles; these alleles have to be distinguished from each other by additional probes. The SSO probe CRX15 ("R-E") specifically distinguishes Dw13 from Dw14 and Dw15, and CRX61 ("S") specifically distinguishes Dw15 from Dw14. The "G" versus "V" polymorphism at position 86 that distinguishes various DRB1 alleles (e.g.,
15 Dw14.1 or DRB1*0404 from Dw14.2 or DRB1*0408) is detected using the CRX56 probe ("G") and the CRX57 probe ("V") (see Table 5).

HTCs with DR types 1 through 10 and the five DR4 Dw subtypes were amplified with the DRB1 specific primers GH46/CRX37, applied to six identical nylon
20 filters, and hybridized with HRP-SSO's specific for the Dw type. The results are shown in Figure 4.

To generate the results shown in Figure 4, about 500 ng of HTC genomic DNA were amplified with the DRB1 specific primers GH46/CRX37. Each pair of filters, A and B, were hybridized with the probes shown in Figure 4. The samples are: Row A:
25 (1) No DNA control; (2) DR1 HTC "KAS9003"; (3) DR2 HTC "SCHU"; (4) DR3 HTC "QBL"; (5) DR4 Dw4 HTC "BSM"; (6) DR4 Dw10 HTC "YAR"; (7) DR4 Dw13 HTC "JHA"; (8) DR4 Dw14 HTC "BM92"; (9) DR4 Dw15 HTC "LKT3"; (10) DR5 HTC "SPOO10"; and (11) DRw6 HTC "OMW"; Row B: (1) No DNA control; (2) DR7 HTC "MOU"; (3) DRw8 HTC "SPACH"; (4) DR9 HTC "DKB"; and (5) DRw10 HTC "SHY".

30 After the hybridization and wash steps, the probes were detected by enhanced chemiluminescence. Panel A shows that all but the DR2, DR7, and DRw9 HTCs hybridized to CRX12.

Panel B shows that CRX53 hybridizes specifically to the Dw4 HTC BSM. An alternative probe for typing DR4 Dw4 is DRB163 (SEQ ID NO: 239), using the
35 hybridization and wash conditions given in Example 9. Another alternative probe for typing DR4 Dw4 that gives a much stronger signal at lower stringencies (2X SSPE, 5X

Denhardt's, 0.5% SDS at 42°C for 15 minutes hybridization and 2X SSPE, 0.1% SDS at 42°C for 15 minutes wash) is probe CRX64, shown below.

CRX64 SEQ ID NO: 83 5'-HRP-GAGGATAAGCIGGCC-3'

HRP is horseradish peroxidase. I is inosine, which slightly destabilizes the probe.

5 Panel C shows detection of the Dw10 HTC "YAR" with the SSO CRX06 ("I-DE"). This probe also hybridizes to the HTC "OMW", which is DRw13, and shares this polymorphism.

Panel D shows specific hybridization of CRX15, which distinguishes the Dw13 sample, JHA, from the Dw14 sample BM92 and the Dw15 sample LKT3.

10 Panel E shows hybridization of CRX04 to JHA, BM92, and LKT3, which are Dw13, Dw14, and Dw15, respectively. CRX04 also hybridizes to the DR1 HTC KAS9003, which is DRB1*0101, and shares this polymorphism ("R") with these three Dw-types. The DR4 Dw14 type is inferred from the probe hybridization pattern in which samples do not hybridize to either CRX15 ("R-E") or CRX61 ("S"; panel F),
15 but do hybridize to CRX04 ("R"). Panel F shows hybridization of CRX61 to the Dw15 HTC "LKT3".

Figure 4 shows that, in general, the present invention enables one to distinguish samples that share polymorphic regions recognized by these probes by the pattern of hybridization to the first panel of probes. For example, DR4 Dw types which are
20 positive for the "R" epitope can be distinguished from DR1 by being positive for GH59 ("V-H") and negative for CRX60 ("W-L-F"); DR4 Dw10 can be distinguished from DRw13 by being positive for GH59 and negative for GH56 ("YSTS").

Table 8 shows that the two DR4 subtypes that this system is not capable of distinguishing are the DRB1*0403 and DRB1*0406 alleles. These two alleles will give
25 the same pattern of hybridization with the two panels of probes. DRB1*0406 is identical to DRB1*0403, except for a serine residue at position 37, where DRB1*0403 has a tyrosine residue. The illustrative panels of probes shown above are not capable of detecting this polymorphism, but the use of additional probes provides complete discrimination.

30

Example 6

Subtyping DR3, DR5, DRw6, and DRw8

The subtypes for the "splits" of DR3, DR5, DRw6, and DRw8 can be identified by using probes that distinguish between the various alleles of each haplotype. For example, both DRw17 and DRw18 hybridize to the "K-GR" probe CRX50, but only
35 DRw17 hybridizes to the "Y" probe GH125, which distinguishes DRw17 from DRw18. Likewise, the DRw11 and the DRw12 alleles (which used to be grouped as

DR5) can be distinguished from each other and from the three DRw8 alleles by using a different combination of probes.

DNA amplified with the DRB1 specific PCR primers CRX37/GH46 from HTCs of the "general" serological DR types 1 through 10 along with HTCs of the DR3, DR5, DRw6, DRw8 subtypes, was applied to 12 filters. Because the serological DR types of these samples were already known, only the probes necessary to determine the subtypes of these alleles were used to illustrate this aspect of the invention. The results are shown in Figure 5.

To generate the results shown in Figure 5, about 500 ng of HTC genomic DNA were amplified with the DRB1 specific primers GH46/CRX37. Each pair of filters, A and B, was hybridized with the probes shown. The samples are: Row A: (1) No DNA control; (2) DR1 HTC "KAS9003"; (3) DR2 HTC "SCHU"; (4) DRw17 HTC "QBL"; (5) DRw18 HTC "RSH"; (6) DR4 HTC "BSM"; (7) DRw11 HTC "SPOO10"; (8) DRw12 HTC "HERLUF"; (9) DRw13/DRw14 "KOSE"; (10) DRw14 HTC "AMALA"; (11) DRw13 HTC "SLE"; Row B: (1) No DNA control; (2) DRw13 HTC "HAG"; (3) DR PEV "BAR P"; (4) DR7 HTC "MOU"; (5) DRw8.3 HTC "TAB"; (6) DRw8.1 HTC "ARC"; (7) DRw8.2 HTC "SPL"; (8) DR9 HTC "DKB"; and (9) DRw10 HTC "SHY". The data for the first panel of probes, which shows that GH56 ("YSTS") hybridizes to DR3, DR5, and DRw6, and GH102 ("YSTG") hybridizes to DRw8 is shown in Figure 7 (see Example 7).

After the hybridization and wash steps, the bound probes were detected by enhanced chemiluminescence. By determining the pattern of probes that hybridize to each sample (see Table 8), the samples can be subtyped for specific alleles, as shown in Figure 6. In Figure 6, samples which hybridized to probes are shown with a "+" symbol. Blank cells indicate samples which did not hybridize to a particular probe. Except for "KOSE and BAR P", all the samples were homozygous typing cells.

The hybridization data and DR types for the DR3, DR5, DRw6 and DRw8 samples are shown in Figure 6. The sample "KOSE" was typed serologically as homozygous for the DRw6 haplotype (though this sample has been shown to be Dw9 and Dw19); however, it types as DRB1*1302 (CRX06 + CRX56) and DRB1*1401 (CRX23 + CRX57).

The sample "BAR P" is typed by serology and MLC as DR4 Dw10 and DRw6. Consequently, "BAR P" hybridizes to the probes CRX35 and CRX56 ("F-DR" and "G"), which types it as the newly discovered "DR PEV" DRw6 allele, and to the probes CRX06 and CRX57 ("I-DE" and "V"), reflecting the presence of the DR4 Dw10 allele (DRB1*0402).

Example 7DR Typing of Cell Lines

Many established cell lines do not express the Class II molecules, making it impossible to DR type them serologically. DNA samples from five Class II negative
5 cell lines coded as a blind panel were amplified with the generic primer pair GH46/GH50 and probed with the first panel of probes (Table 4) to establish the general serological DR types for the samples. (Figure 7).

For DR typing these and other cell lines, approximately 100 ng of cell line DNA were amplified for 30 cycles with the generic HLA DRB primers GH46/GH50. Each
10 filter containing amplified cell line DNA (samples A through F) was hybridized simultaneously with a control filter (samples 1 through 11) containing HTC DNAs amplified with the generic HLA DRB primers. All filters were prepared as described in Example 1. The results are shown in Figure 7. Each pair of filters was hybridized to the SSO probes shown. The samples are: (1) No DNA control; (2) DR1 HTC
15 "KAS9003"; (3) DR2 HTC "SCHU"; (4) DR3 HTC "QBL"; (5) DR4 HTC "BSM"; (6) DR5 (DRw11) HTC "SPOO10"; (7) DRw6 HTC "OMW"; (8) DR7 HTC "MOU"; (9) DRw8 HTC "SPACH"; (10) DR9 HTC "DKB"; (11) DRw10 HTC "SHY;" (A) No DNA control; (B) Raji; (C) 616; (D) Beguit; (E) RM3; and (F) RS225.

The signal intensity for the CRX12 probe demonstrates that all of the samples
20 were equally well amplified. Raji, RM3, and RS225 hybridize to the probe CRX34, which is specific for DRw10. Sample 616 hybridizes to the probes CRX60 ("WLF") and CRX04 ("R"), which is consistent with this sample having the DR1 allele.

The cell line Beguit hybridizes to the DR7 probe CRX49 ("G-YK") and to two other probes, GH56 ("YSTS") and GH122 ("E"), suggesting that it is DR7 and
25 DRw11. However, the putative DRw11 allele from this sample would be expected to hybridize either to CRX35 ("F-DR"; DRB1*1101) or CRX06 ("I-DE"; DRB1*1102), but it does not, suggesting that it may be a new variant of DRw11, as discussed further below.

This sample, derived from an individual with bare lymphocyte syndrome, was
30 cloned and sequenced to determine the nature of the polymorphism at this position. Sequencing of the allele showed that there is indeed a sequence polymorphism at the third hypervariable region that codes for the epitope "F-DE"; this allele types as DRB1*1103 (see sequence "Beguit" in Table 3). Probe CRX68 hybridizes specifically to this polymorphism in the third hypervariable region (see Table 4).

35 All of the other samples also hybridize to the GH56 "YSTS" probe, so they could be DR3, DRw11, or DRw6. Because these other samples do not hybridize to the "E" probe, it is likely they are DR3 or DRw6. However, the samples do not hybridize

to the probes CRX06 ("I-DE") or CRX23 ("A-H"), which recognize sequences present in three common DRw6 alleles, DRB1*1301, DRB1*1302, and DRB1*1401; this suggests that they are not DRw6, but DR3.

To confirm this, the samples were amplified with the DRB1 specific primers
5 GH46/CRX37 and probed with the SSOs for DR3, GH125 ("Y") and CRX50 ("K-GR"). About 100 ng of cell line genomic DNA were amplified with the DRB1 specific primers GH46/CRX37 as described in Example 1. Each filter containing amplified cell line DNA (samples A through F) was hybridized simultaneously with a control filter (samples 1 through 11) containing HTC DNAs amplified with the DRB1 specific
10 primers. All filters were prepared as described in Example 1. The results are shown in Figure 8.

Each pair of filters was hybridized the SSO probes shown. The samples are:
(1) No DNA control; (2) DR1 HTC "KAS9003"; (3) DR2 HTC "SCHU"; (4) DR3
HTC "QBL"; (5) DR4 HTC "BSM"; (6) DR5 (DRw11) HTC "SPOO10"; (7) DRw6
15 HTC "OMW"; (8) DR7 HTC "MOU"; (9) DRw8 HTC "SPACH"; (10) DR9 HTC
"DKB"; (11) DRw10 HTC "SHY"; (A) No DNA control; (B) Raji; (C) 616; (D)
Beguit; (E) RM3; and (F) RS225. After the hybridization and wash steps, the bound probes were detected by enhanced chemiluminescence.

As Figure 8 shows, Raji, 616, RM3, and RS225 hybridize to both the "Y" and
20 "K-GR", which types them as DR3 (DRw17). In summary, Raji, RM3, and RS225
type as DRw10/DRw17. RM3 and RS225 are cell lines derived from Raji, so it is not
surprising that they have the same DRB type. Cell line 616 types as DR1/DRw17, and
Beguit types as DR7/DRw11 but, as noted above, the Beguit cell line contains the
DRB1*1103 allele, which has the "F-DE" epitope. Previously, most DRw11 typing
25 cells were observed to contain DRB1 alleles with the "F-DR" epitope. Thus, the Beguit
cell line contains an unusual DRB1 allele for the DRw11 type.

In addition to cell line samples, DNA from three different sources was amplified
and typed. One source is purified genomic DNA from a set of unrelated, heterozygous
individuals from the Center for Study of Human Polymorphism (CEPH, Paris, France;
30 samples 555 to 863). Another source was cDNA made from mRNA of cancer tissue
from patients with bladder carcinoma (samples 2426, 2446, 2540, 2671, 2755).
Normal human bladder cells do not express Class II molecules, but various carcinoma
cells have been reported as expressing Class II molecules.

The remaining sample (PSW) is a buccal swab amplified for HLA DRB directly
35 without DNA purification. For complete DR typing, the samples were amplified with
both the generic DRB primers GH46/50 and the DRB1 specific PCR primers
GH46/CRX37. Filter strips containing DNA samples amplified with the general probes

were probed with the first panel of probes (Table 4), and filter strips containing DNA samples amplified specifically for DRB1 were probed with the second panel of probes (Table 5).

5 Comparing the pattern of hybridization with Table 3 and Table 8, one can unambiguously derive the DRB-type of the samples. The results for the typing of the heterozygous samples are shown in Figure 9. In Figure 9, samples which hybridized to a probe are shown with a "+" symbol. Blank cells indicate samples which did not hybridize to a particular probe.

10 Samples 555 to 863 are pure genomic DNA provided by CEPH. Sample "PSW" is a sample amplified directly from a buccal swab. This sample was heated for 5 minutes at 95°C in 200 µl of 5% Chelex (Singer-Sam *et al.*, 1989, Amplifications 3:11). About 50 µl of this solution were amplified directly in 200 µl of reaction volume, and generic DRB and DRB1 specific amplification reactions were conducted for the sample for 40 cycles.

15 Samples 2426, 2446, 2540, 2671, and 2755 were amplified from bladder carcinoma cDNA preparations. The bladder carcinoma samples 2426, 2446, 2540, 2671, and 2755 were not analyzed with the SSOs GH125, CRX50, CRX56, and GH54, because these probes require amplification with the DRB1 specific primers GH46/CRX37. Because CRX37 is derived from intron sequence, it cannot be used to
20 amplify cDNA. ND is not determined.

The buccal swab sample, PSW, is DR4 Dw14 (DRB1*0404) and DRw11.

These data show that the system is capable of typing heterozygous DNAs from a variety of sources, from standard purified genomic DNA, from cDNAs synthesized from RNA, and from unusual sources, such as a buccal swab or single hair.

25

Example 8

DRB Typing - Reverse Dot Blot Format

In this embodiment of the invention, the DRB probes are fixed to a membrane, and the amplified target DNA is hybridized to the membrane-bound probe. The set of typing probes is designed so that each probe will hybridize to a specific target sequence
30 at the same temperature and salt concentration (and stay hybridized under the same wash conditions) as all other probes in the set. The PCR primers used in the amplification are biotinylated, as described in the book PCR Protocols, incorporated herein by reference, so that any amplified DNA that hybridizes to the membrane-bound probes can be easily detected.

35

In one embodiment, detection is carried out by reacting streptavidin (SA)-conjugated horseradish peroxidase with any biotinylated, amplified DNA hybridized to

the membrane-bound probe. The HRP thus becomes bound, through the SA-biotin interaction, to the amplified DNA and can be used to generate a signal by a variety of well known means, such as the generation of a colored compound, e.g., by the oxidation of tetramethylbenzidine (see U.S. Patent No. 4,789,630, incorporated herein
5 by reference).

Although the probes can be fixed to the membrane by any means, a preferred method involves "tailing" an oligonucleotide probe about 13 to 25 nucleotides in length with a much longer sequence of poly-dT. The resulting poly-dT "tail" can then be reacted with amine groups on the membrane to fix the probe covalently to the
10 membrane. This reaction can be facilitated by UV irradiation.

Terminal deoxyribonucleotidyl transferase (TdT, Ratliff Biochemicals; for the reactions below assume a concentration of about 120 Units/ μ l, which is 100 pmol/ μ l) can be used to create a poly-dT tail on a probe, although one can also synthesize the tailed probe on a commercially available DNA synthesizer. When one uses a DNA
15 synthesizer to make the tailed probe, however, one should place the tail on the 5' end of the probe, so that undesired premature chain termination occurs primarily in the tail region.

TdT reactions should be carried out in volume of about 100 μ l containing 1X TdT salts, 200 pmol of oligonucleotide, 800 μ M dTT, and 60 units of TdT. 10X TdT
20 salts is 1,000 mM K-cacodylate, 10 mM CoCl_2 , 2 mM dithiothreitol, 250 mM Tris-Cl, pH 7.6, and is prepared as described by Roychoudhury and Wu, Meth. Enzymol. 65:43-62, incorporated herein by reference. A 10X stock solution of 8 mM dTTP can be prepared (neutralized to pH 7 with NaOH) for convenience.

The TdT reaction should be carried out at 37°C for two hours and then stopped
25 by the addition of 100 μ l of 10 mM EDTA, pH 8. The final concentration of tailed oligonucleotide is 1 μ M (1 pmol/ μ l), and the length of the homopolymer tail is about 400 residues. Tail length can be changed by adjusting the molar ratio of dTTP to oligonucleotide. The tailed probes can be stored at -20°C until use.

Two types of nylon membrane are preferred for the reverse dot blot format:
30 Biodyne™ nylon membrane, 0.45 micron pore size, manufactured by Pall; and Biotrans™ nylon membrane, 0.45 micron pore size, manufactured by ICN. The probes can be spotted onto the membrane very conveniently with the Bio-Dot™ dot blot apparatus manufactured by BioRad. Each probe is spotted onto a unique, discrete location onto the membrane. About 5 to 10 picomoles of each tailed probe is premixed
35 with 60-100 μ l of TE buffer before application to the dot blot apparatus. After dot blotting, the membrane is briefly placed on absorbent paper to draw off excess liquid.

The membrane is then placed inside a UV light box, such as the Stratalinker™ light box manufactured by Stratagene, and exposed to 50 to 60 millijoules of flux to fix the tailed probe to the nylon membrane. After a brief rinse (for about 15 minutes in hybridization solution) to remove unbound probe, the membrane is then ready for

5 hybridization with biotinylated PCR product. One-half to one picomole (one-quarter to one-half of a typical, 100 µl PCR mixture) of PCR product is added to each probe panel for hybridization. About 50 µl of streptavidin-horseradish peroxidase (SA-HRP, commercially available from PECL; see the instruction manual for the AmpliType™ DQα DNA Typing Kit, incorporated herein by reference) conjugate can be added at this

10 time for convenience, but better signals will result if a separate SA-HRP incubation and wash, at room temperature, is performed after the stringency wash.

Hybridization is typically carried out at 50°C for 30 minutes in a water bath and with hybridization buffer composed of 0.5% SDS and 3X to 5X SSPE, most commonly 4X. Stringency washing is carried out at 50°C for 15 minutes in a water

15 bath and with wash solution composed of 0.1% SDS and 1X SSPE. A post-wash of 1X PBS at room temperature for 30 minutes can enhance signal quality.

The biotinylated primers for the reverse dot blot method and other useful primers of the invention are shown below. Note, however, that one or both of the primers can be biotinylated in an amplification and that the primers can be used for

20 amplification with any detection format.

Primer	Seq. ID No.	Sequence
CRX11	SEQ ID NO: 62	5'-TCTAGAAGTACTCTACGTCT-3'
CRX28	SEQ ID NO: 67	B-CCGGATCCTTCGTGTCCCCACAGCACG-3'
CRX29	SEQ ID NO: 68	B-CTCCCCAACCCCGTAGTTGTGTCTGCA-3'
25 DRB17	SEQ ID NO: 73	B-GAATTCCCGCGCCGCGCT-3'
DRB30	SEQ ID NO: 107	B-GAATTCCCGCGCCGCGCTCACCT-3'
DRB152	SEQ ID NO: 228	B-CCCCGTAGTTGTGTCTGCACACGG-3'
DB259	SEQ ID NO: 95	B-GAATTCCCGCGCCGCGCTCACCTCG-3'
DB260	SEQ ID NO: 96	B-GAATTCCCGCGCCGCGCTCACCTCGCC-3'

30 B is biotin. CRX11 is a left-end primer designed for use with GH50 to amplify the DRB1 second exon of DR3, DR5, and DRw6 (13 and 14) haplotypes. CRX28 is biotinylated left-end primer GH46; CRX29 is biotinylated right-end primer GH50; and DRB17 is biotinylated right-end primer CRX37. DRB30 is a right-end primer that includes the CRX37 sequence and has the same DRB1 range as CRX37 except that,

35 unlike CRX37, DRB30 can amplify DR7 DRB1 sequences. DRB152 is a group specific right-end primer for DR7 and DR9 alleles. DB259 and DB260 are also right-

end primers designed to extend the range of DRB1 specific amplification to DR2 and DR9.

The hybridizing regions of the tailed probes for use in the reverse dot blot method are shown below. X is inosine. Where two probe names are shown, i.e.,

- 5 DRB01/CRX60, the first name designates the hybridizing region (shown) of the tailed probe, the second designates the HRP-labeled, untailed probe.

	<u>Primer</u>	<u>Seq. ID No.</u>	<u>Sequence</u>
	CRX23	SEQ ID NO: 66	S 5'-CCTGCTGCGGAGCACTG
	GH54	SEQ ID NO: 85	U 5'-GCTGTTCCAGGACTC
10	GH56	SEQ ID NO: 86	S 5'-CAGACGTAGAGTACTCC
	GH59	SEQ ID NO: 87	U 5'-CATGTTTAACCTGCTCC
	GH102	SEQ ID NO: 89	S 5'-GAAATAAACACTCACCCGTAG
	GH104	SEQ ID NO: 90	S 5'-TGACACTCCCTCTTAGGCT
	GH105	SEQ ID NO: 91	U 5'-CTTGCAGCAGGATAAGTATG
15	GH111	SEQ ID NO: 92	S 5'-TTGAAGCAGGATAAGTTTGA
	GH122	SEQ ID NO: 93	S 5'-CAGTACTCCTCATCAGG
	GH125	SEQ ID NO: 94	U 5'-CTGTCCAGGTACCGCAC
	DRB01/CRX60	SEQ ID NO: 79	S 5'-CAAACCTTAAGCTGCCAC
	DRB02/CRX06	SEQ ID NO: 61	S 5'-CATCCTGGAAGACGAGC
20	DRB03/CRX35	SEQ ID NO: 71	U 5'-CCTGTCTTCCAGGAAGT
	DRB04/CRX49	SEQ ID NO: 74	U 5'-TGACACTTATACTTACC
	DRB05/CRX34	SEQ ID NO: 70	U 5'-CTCAAACCTTAACCTCCTC
	DRB06/CRX04	SEQ ID NO: 60	U 5'-GAGCAGAGGCGGGCC
	DRB07/CRX68	SEQ ID NO: 84	S 5'-GACTTCCTGGAAGACGA
25	DRB08/CRX12	SEQ ID NO: 63	U 5'-AGCTGGGGCGGCCT
	DRB09/CRX50	SEQ ID NO: 75	U 5'-CACCCGGCCCCGCTTCT
	DRB10/CRX53	SEQ ID NO: 76	U 5'-GAGCAGAAGCGGGCC
	DRB11/CRX15	SEQ ID NO: 64	U 5'-ACCTCGGCCCCGCCTC
	DRB12/CRX62	SEQ ID NO: 81	U 5'-ACATCCTGGAAGACAAG
30	DRB13/CRX63	SEQ ID NO: 82	U 5'-ACATCCTGGAAGACAGG
	DRB14/CRX61	SEQ ID NO: 80	U 5'-GCGGCCTAGCGCCGAGT
	DRB15/CRX56	SEQ ID NO: 77	S 5'-CGGGTTGGTGAGAGCT
	DRB16/CRX57	SEQ ID NO: 78	S 5'-CGGGTTGTGGAGAGCT
	DRB19	SEQ ID NO: 97	S 5'-TGACACTTATACTTACCGTC
35	DRB20	SEQ ID NO: 98	S 5'-CTCAAACCTTAACCTCCTCC
	DRB21	SEQ ID NO: 99	S 5'-GAGCAGAGGCGGGC
	DRB22	SEQ ID NO: 100	U 5'-GCCTGTCTTCCAGGAAGT

	DRB23	SEQ ID NO: 101	U 5'-GCCCGCTTCTGCTC
	DRB24	SEQ ID NO: 102	U 5'-GAGCAGAAGCXGGCC
	DRB25	SEQ ID NO: 103	U 5'-CATCCTGGAAGACAGG
	DRB26	SEQ ID NO: 104	U 5'-CATCCTGGAAGACAGGCGCG
5	DRB27	SEQ ID NO: 105	S 5'-ACATCCTGGAAGACAAGC
	DRB28	SEQ ID NO: 107	U 5'-ACCTCGGCCCXCCTC
	DRB31	SEQ ID NO: 108	S 5'-CTCCCGTTTATGGATGTATC
	DRB32	SEQ ID NO: 109	S 5'-TGTCCAGGTACCGCA
	DRB33	SEQ ID NO: 110	S 5'-ACTCATGTTTAACCTGCTCC
10	DRB34	SEQ ID NO: 111	S 5'-CTCATGTTTAACCTGCTCC
	DRB35	SEQ ID NO: 112	S 5'-TGTCGCCGAGTCCTGG
	DRB36	SEQ ID NO: 113	S 5'-CGCCTGTCTTCCAGGAAGT
	DRB37	SEQ ID NO: 114	S 5'-CCGCCTGTCTTCCAGGAAGT
	DRB38	SEQ ID NO: 115	U 5'-TCCACCCGGCCCCGCTTCT
15	DRB39	SEQ ID NO: 116	U 5'-GAGCAGAAGCGGGC
	DRB40	SEQ ID NO: 117	U 5'-GAGXAGAAGCXGGCC
	DRB41	SEQ ID NO: 118	U 5'-GGCCCGCTTCTGCTC
	DRB42	SEQ ID NO: 119	S 5'-GACGGAGCTGGGGCGGCCT
	DRB43	SEQ ID NO: 120	U 5'-GACTTCCTGGAGCGGAGG
20	DRB44	SEQ ID NO: 121	U 5'-GACCTCCTGGAGCGGAGG
	DRB45	SEQ ID NO: 122	S 5'-GAGCGGAGGCGTGCC
	DRB46	SEQ ID NO: 123	S 5'-TCAGACGTAGAGTACTCC
	DRB47	SEQ ID NO: 124	U 5'-TCTTGCAGCAGGATAAGTATG
	DRB48	SEQ ID NO: 125	S 5'-CACTCATGTTTAACCTGCTCC
25	DRB49	SEQ ID NO: 126	U 5'-TCAGACTTACGCAGCTCC
	DRB50	SEQ ID NO: 127	U 5'-TCAGACTTAAGCAGCTCC
	DRB51	SEQ ID NO: 128	U 5'-GAGCAGAGGCGGAGC
	DRB52	SEQ ID NO: 129	U 5'-GAGCAGAAGCGAGGCC
	DRB53	SEQ ID NO: 130	U 5'-GGCCCGCTTCTGCTCCA
30	DRB54	SEQ ID NO: 131	U 5'-GCGGCCCCGCTTCTGCTC
	DRB55	SEQ ID NO: 132	U 5'-GAGCAGAAGCGAGGC
	DRB56	SEQ ID NO: 133	U 5'-GGAGXAGAAGCXGGCCG
	DRB57	SEQ ID NO: 134	U 5'-CCACCCGGCCCCGCTTCT
	DRB58	SEQ ID NO: 135	U 5'-CATCCTGGAAGACAGGCG
35	DRB59	SEQ ID NO: 136	U 5'-CATCCTGGAAGACAGAGCG
	DRB60	SEQ ID NO: 137	S 5'-GGCGGCCTAGCGCCGAGT
	DRB61	SEQ ID NO: 138	U 5'-ACCTCCTGGAGCGGAGG

	DRB62	SEQ ID NO: 139	S	5'-GACTTCCTGGAGCGGAG
	DRB63	SEQ ID NO: 140	S	5'-CATCCTGGAGCAGGCG
	DRB64	SEQ ID NO: 141	U	5'-ACCTCGGCCCXCCTCTG
	DRB66	SEQ ID NO: 142	U	5'-GAGCAGAAGCGGG
5	DRB67	SEQ ID NO: 143	U	5'-GAGXAGAAGCXGGCCG
	DRB68	SEQ ID NO: 144	U	5'-CCTCGGCCCXCCTCTGC
	DRB69	SEQ ID NO: 145	U	5'-CCTCCTGGAGCGGAGG
	DRB70	SEQ ID NO: 146	U	5'-CGCCTGTCTTCCAGGATG
	DRB71	SEQ ID NO: 147	U	5'-TTCTTGCAGCAGGATAAGTATG
10	DRB72	SEQ ID NO: 148	S	5'-CGCCTGTCCTCCAGGATG
	DRB73	SEQ ID NO: 149	U	5'-AGAAGCGGGGCCGGGTG
	DRB74	SEQ ID NO: 150	U	5'-GAGCAGAGXCGGGCC
	DRB75	SEQ ID NO: 151	U	5'-GAGCAGAGAGCGGGC
	DRB76	SEQ ID NO: 152	U	5'-CTTCTGCTCCAGGAGG
15	DRB77	SEQ ID NO: 153	U	5'-CTCCTGGAGCAGAAG
	DRB78	SEQ ID NO: 154	U	5'-CCTCCTGGAGCXGAAG
	DRB79	SEQ ID NO: 155	U	5'-CCTCCTGGAGCAAGAAG
	DRB80	SEQ ID NO: 156	U	5'-CGGCCCCGCCTCTGCTC
	DRB81	SEQ ID NO: 157	U	5'-CGGGGCTGTGGAGAGCT
20	DRB82	SEQ ID NO: 158	U	5'-CTTCTGCTCCAGGAGGTC
	DRB83	SEQ ID NO: 159	U	5'-ACCTCCTGGAGCAGAAG
	DRB84	SEQ ID NO: 160	S	5'-GGCCCCGCCTCTGCTC
	DRB85	SEQ ID NO: 161	U	5'-GCCCCGCCTCTGCTC
	DRB86	SEQ ID NO: 162	U	5'-GGCCCCGCCTCTGC
25	DRB87	SEQ ID NO: 163	U	5'-GAGGCGCGCCGAGGT
	DRB88	SEQ ID NO: 164	U	5'-GAGGCGCGCCGAGGTG
	DRB89	SEQ ID NO: 165	U	5'-GAGGCGCGCCGAGGTGGA
	DRB90	SEQ ID NO: 166	S	5'-AGAAGCGGGGCCGGGT
	DRB91	SEQ ID NO: 167	S	5'-AGAAGCGGGGCCGGG
30	DRB92	SEQ ID NO: 168	U	5'-GGGGTTGGTGAGAGCT
	DRB93	SEQ ID NO: 169	U	5'-GGGGTTGTGGAGAGCT
	DRB94	SEQ ID NO: 170	U	5'-ACCTCGGCCCCGCCTC
	DRB95	SEQ ID NO: 171	S	5'-CATCCTGGAAGACAGGC
	DRB96	SEQ ID NO: 172	U	5'-GAGCAGAAGCAGGCC
35	DRB97	SEQ ID NO: 173	U	5'-GGCGGCCTAGCGCCGAGTAC
	DRB98	SEQ ID NO: 174	U	5'-TGTAGGACCTTCTGTCCG
	DRB99	SEQ ID NO: 175	U	5'-GTAGGACCTTCTGTCCG

	DRB100	SEQ ID NO: 176	S	5'-TTCTTGCAGCAGGATAAGTATGAG
	DRB101	SEQ ID NO: 177	S	5'-CTCCCGTTTATGGATGTATC
	DRB102	SEQ ID NO: 178	S	5'-CAGTACTCCTCATCAGGC
	DRB103	SEQ ID NO: 179	S	5'-CTGTCCAGGTACCGCA
5	DRB104	SEQ ID NO: 180	U	5'-ACCTCGGCCCGCCTCT
	DRB105	SEQ ID NO: 181	U	5'-GAGGCGCGCCGAGGTGGAC
	DRB106	SEQ ID NO: 182	U	5'-AGAGGCGCGCCGAGGTGGAC
	DRB107	SEQ ID NO: 183	S	5'-AGAAGCGGGGCCGG
	DRB108	SEQ ID NO: 184	S	5'-GAAGCGGGGCCGGG
10	DRB109	SEQ ID NO: 185	S	5'-GAAGACAGGCGGGCCCTGG
	DRB110	SEQ ID NO: 186	U	5'-GCCTGTCTTCCAGGAAGTCC
	DRB111	SEQ ID NO: 187	U	5'-CTCAGACGTAGAGTACTCC
	DRB112	SEQ ID NO: 188	S	5'-GCCTGCTGCGGAGCACTGG
	DRB113	SEQ ID NO: 189	S	5'-GACCTCCTGGAAGACAGG
15	DRB114	SEQ ID NO: 190	U	5'-CCTGTCCTCCAGGAGGTC
	DRB115	SEQ ID NO: 191	U	5'-ACGGGGTTGGTGAGAGCTT
	DRB116	SEQ ID NO: 192	U	5'-ACGGGGTTGTGGAGAGCTT
	DRB117	SEQ ID NO: 193	U	5'-ACGGGGCTGTGGAGAGCTT
	DRB118	SEQ ID NO: 194	S	5'-GAGGCGGGCCGAGGT
20	DRB119	SEQ ID NO: 195	U	5'-GAGGCGGGCCGAGGTG
	DRB120	SEQ ID NO: 196	U	5'-GAGGCGGGCCGAGGTGGA
	DRB121	SEQ ID NO: 197	U	5'-GAGGCGGGCCGAGGTGGAC
	DRB122	SEQ ID NO: 198	U	5'-AGAGGCGGGCCGAGGTGGAC
	DRB123	SEQ ID NO: 199	T	5'-GGCGGCCTAGCGCCGAGTA
25	DRB124	SEQ ID NO: 200	T	5'-CCACXCGGCCCGCTTCT
	DRB125	SEQ ID NO: 201	T	5'-GAGGCGGGCCGCGGT
	DRB126	SEQ ID NO: 202	T	5'-ACCGCGGCCCGCCTC
	DRB127	SEQ ID NO: 203	T	5'-GAAGCGGGCCGCGGT
	DRB128	SEQ ID NO: 204	T	5'-ACCGCGGCCCGCTTC
30	DRB129	SEQ ID NO: 205	T	5'-ACTTCCTGGAAGACAGG
	DRB130	SEQ ID NO: 206	T	5'-CGCAAGTCCTCCTCTTG
	DRB131	SEQ ID NO: 207	T	5'-CAAGAGGAGGACTTGCG
	DRB132	SEQ ID NO: 208	T	5'-GAAGACAGGCGGGCCCTG
	DRB133	SEQ ID NO: 209	T	5'-AAGACAGGCGGGCCCTGG
35	DRB134	SEQ ID NO: 210	T	5'-ACTTCCTGGAAGACGAG
	DRB135	SEQ ID NO: 211	T	5'-ACTTCCTGGAAGACGAGC
	DRB136	SEQ ID NO: 212	T	5'-ACATCCTGGAAGACAGGC

	DRB137	SEQ ID NO: 213	T	5'-GCCTGTCTTCCAGGATG
	DRB138	SEQ ID NO: 214	T	5'-GCAGAAGCGGGCCGCG
	DRB139	SEQ ID NO: 215	T	5'-CGCGGCCCCGCTTCTGC
	DRB140	SEQ ID NO: 216	T	5'-GCAGAGGCGGGCCGCG
5	DRB141	SEQ ID NO: 217	T	5'-CGCGGCCCCGCTTCTGC
	DRB142	SEQ ID NO: 218	T	5'-GCAGAGGCGGGCCGAG
	DRB143	SEQ ID NO: 219	T	5'-CTCGGCCCCGCTTCTGC
	DRB144	SEQ ID NO: 220	T	5'-GCGGAGGCGGGCCGAG
	DRB145	SEQ ID NO: 221	T	5'-CTCGGCCCCGCTCCGC
10	DRB146	SEQ ID NO: 222	T	5'-CTCCGCTCCAGGAAGTC
	DRB147	SEQ ID NO: 223	T	5'-CGGGGTTGGTGAGAGCT
	DRB148	SEQ ID NO: 224	T	5'-CGGGGTTGTGGAGAGCT
	DRB149	SEQ ID NO: 225	T	5'-CGGGGCTGTGGAGAGCTT
	DRB150	SEQ ID NO: 226		5'-TGTCCACCGCGGCCCCGCGCCT
15	DRB153	SEQ ID NO: 229		5'-GAATTCCCAGCTCACACGGGACT
	DRB154	SEQ ID NO: 230		5'-GGTGTCCACCGCGGCCCCGCGC
	DRB155	SEQ ID NO: 231		5'-AACCCCGTAGTTGTGTCTGCACAC
	DRB156	SEQ ID NO: 232		5'-GGGGGAGTTCCGGG
	DRB157	SEQ ID NO: 233		5'-CCCGGTACTCCCCC
20	DRB158	SEQ ID NO: 234		5'-CGCGGCCCCGCTTCTG
	DRB159	SEQ ID NO: 235		5'-CCGCGGCCCCGCTTCTG
	DRB160	SEQ ID NO: 236		5'-CCGXGGCCCCGCTTCTGC
	DRB161	SEQ ID NO: 237		5'-CCAGCGGCCCCGCTTCTGC
	DRB162	SEQ ID NO: 238		5'-GCAGAAACGGGCGCGXGT
25	DRB163	SEQ ID NO: 239		5'-GCAGAAAGCGGGCCGCGXGT
	DRB164	SEQ ID NO: 240		5'-CAGAAGCGGGCCGCG
	DRB165	SEQ ID NO: 241		5'-ACCTXGGCCCCGCCXCTGC
	DRB166	SEQ ID NO: 242		5'-ACCTXGGCCCCGCCXCTG
	DRB167	SEQ ID NO: 243		5'-GGAGCAGAAACGGGCCC
30	DRB168	SEQ ID NO: 244		5'-GGAGCAGAAACGGGCCCC
	DRB169	SEQ ID NO: 245		5'-GCAGAAAGCGGGCCXCG
	DRB170	SEQ ID NO: 246		5'-GTCCACCTCGGCCCC
	DRB171	SEQ ID NO: 247		5'-CGGGCCGCGGTGGAC
	DRB172	SEQ ID NO: 248		5'-CGCCTCCGCTCCAGGAG
35	DRB173	SEQ ID NO: 249		5'-CTCCTGGAGCAGAGGCG
	DRB174	SEQ ID NO: 250		5'-ACCGCGGCCCCGCTTCT
	DRB175	SEQ ID NO: 251		5'-CACCTXGGCCCCGCCXCTG

	DRB176	SEQ ID NO: 252	5'-CGXGGCCCCGCCTCTG
	DRB177	SEQ ID NO: 253	5'-CCGXGGCCCCGCCTCTG
	DRB178	SEQ ID NO: 254	5'-GGGGGAGTTCCGGGCG
	DRB179	SEQ ID NO: 255	5'-CGCCCGGTACTCCCCC
5	DRB180	SEQ ID NO: 256	5'-XCCTGATGCCGAGTACTG
	DRB181	SEQ ID NO: 257	5'-XCGGGGCTGTGGAGAGCTT
	DRB182	SEQ ID NO: 258	5'-CTACGGGGCTGTGGAGAG
	DRB183	SEQ ID NO: 259	5'-CTACGGGXCTGTGGAGAG
	DRB184	SEQ ID NO: 260	5'-GTTCCGGGCGGTGAC
10	DRB185	SEQ ID NO: 261	5'-GGGGGAGTTXCGGGG
	DRB186	SEQ ID NO: 262	5'-CGTCACCGCCCGGTAC
	DRB187	SEQ ID NO: 263	5'-CGTCACCGCCCGXTAC
	DRB188	SEQ ID NO: 264	5'-CACCCCTCATXGCCC
	DRB189	SEQ ID NO: 265	5'-GCGGGCCGCGGTGGAC
15	DRB190	SEQ ID NO: 266	5'-CAGAGGCXGGCCGCGGT
	DRB191	SEQ ID NO: 267	5'-GTTXCGGGCGGTGAC
	DRB192	SEQ ID NO: 268	5'-TTCCGGGCGGTGAC
	DRB193	SEQ ID NO: 269	5'-GGGGAGTTCCGGG
	DRB194	SEQ ID NO: 270	5'-TCACCGCCCGGAAC
20	DRB195	SEQ ID NO: 271	5'-AGATACTTCTATAACCAG
	DRB196	SEQ ID NO: 272	5'-AGACACTTCTATAACCAG
	DRB197	SEQ ID NO: 273	5'-CTGGTTATAGAAGTATCT
	DRB198	SEQ ID NO: 274	5'-CTGTCGCCGAGTCCTGG
	DRB199	SEQ ID NO: 275	5'-GGGCGGCCTAGCGCCGAGT
25	DRB200	SEQ ID NO: 276	5'-GCAGAAAGCGGGCCGCXGT
	DRB201	SEQ ID NO: 277	5'-GCGXCTGTCTTCCAGGATG
	DRB202	SEQ ID NO: 278	5'-CGXCTGTCTTCCAGGATG
	DRB203	SEQ ID NO: 279	5'-ACCGXGGCCCCGCCTCTG
	DRB204	SEQ ID NO: 280	5'-CCGTCACCGCCCGXTAC
30	DRB205	SEQ ID NO: 281	5'-GGGGAGTTCCGGGG
	DRB206	SEQ ID NO: 282	5'-TCACCGCCCGGAACTC
	DRB207	SEQ ID NO: 283	5'-TGACACTTATACTTACCCTGC
	DRB208	SEQ ID NO: 284	5'-TTGAAGCAGGATAAGTTTGAG
	DRB209	SEQ ID NO: 285	5'-CTTGAAGCAGGATAAGTTTG
35	DRB210	SEQ ID NO: 286	5'-GAATTCCCGCGCCGCGCTCA
	DRB211	SEQ ID NO: 287	5'-GAATTCCCGCGCCGCG
	DRB212	SEQ ID NO: 288	5'-GAATTCCCGCGCCGCGCTCAC

	DRB213	SEQ ID NO: 289	5'-ATGACACTCCCTCTTAGGCTG
	DRB214	SEQ ID NO: 290	5'-ACATCCTGGAAGACGAG
	DRB215	SEQ ID NO: 291	5'-CCGCTCCGTCCCATTGAA
	DRB216	SEQ ID NO: 292	5'-TTCAATGAGACGGAGCGG
5	DRB217	SEQ ID NO: 293	5'-CATCCTGGAAGACGAG
	DRB218	SEQ ID NO: 294	5'-GCTCGTCTTCCAGCATG
	DRB219	SEQ ID NO: 295	5'-CGCTCGTCTTCCAGGATG
	DRB220	SEQ ID NO: 296	5'-GCTGTCGCCGAGTCCTGG
	DRB221	SEQ ID NO: 297	5'-CCTGTCGCCGAGTCCTGG
10	DRB222	SEQ ID NO: 298	5'-CTGTCCAGGTACCGCA
	DRB223	SEQ ID NO: 299	5'-GGCGGCCTAGCGCCGAGTA
	DRB224	SEQ ID NO: 300	5'-CCACXCGGCCCGCTTCT
	DRB225	SEQ ID NO: 301	5'-GAGGCGGGCCGCGGT
	DRB226	SEQ ID NO: 302	5'-ACCGCGGCCCGCCTC
15	DRB227	SEQ ID NO: 303	5'-GAAGCGGGCCGCGGT
	DRB228	SEQ ID NO: 304	5'-ACCGCGGCCCGCTTC
	DRB229	SEQ ID NO: 305	5'-ACTTCCTGGAAGACAGG
	DRB230	SEQ ID NO: 306	5'-GACCTCCTGGAAGACAGG
	DRB231	SEQ ID NO: 307	5'-ACATCCTGGAAGACAAGC
20	DRB232	SEQ ID NO: 308	5'-GACATCCTGGAAGACAAGC
	DRB233	SEQ ID NO: 309	5'-ACATCCTGGAAGACAAGCG
	DRB300	SEQ ID NO: 310	5'-GAATTCCCGCGCCGCTCACCTC
	DRB305	SEQ ID NO: 311	5'-GAATTCACAGGGACTCCAGGCC

25 Preliminary testing shows that some probes, marked "S" above are more preferred than other probes, marked "U" above. Other probes, marked "T" above, have not yet been tested.

30 The hybridization patterns and specificity (1989 allele set) for the preferred reverse dot blot probes are show below. Where an "X" is used in the "specificity" column, the "X" designates inclusivity of all alleles indicated by the first two digits after the "*".

	<u>Epitope</u>	<u>Name</u>	<u>Specificity</u>
	W-L-F	CRX60DRB01	DRB1*0101, 0102, 0103
	W-P-R	GH104	DRB1*1501, 1502, 1601, 1602
	QDY	DRB100	DRB5*0101, 0102, 0201, 0202
5	K-D-F	GH111	DRB1*0901
	YSTS	DRB46	DRB1*030X, 110X, 130X, 140X
	YSTG	GH102	DRB1*080X, 1201
	V--H	DRB48	DRB1*040X
	G-YK	DRB19	DRB1*070X
10	EV	DRB20	DRB1*1001
	LR-S	GH57	DRB3*0101
	LL-S	GH58	DRB3*0201, 0202, 0301
	F--DR	DRB37/DRB109	DRB1*0801, 0802, 1101, 1104, 1601, PEV; DRB5*0101, 0102
15	F--DE	CRX68	DRB1*1103
	I--DE	CRXO6/DRB02	DRB1*0103, 0402, 1102, 1301, 1302
	I--DK	DRB27	DRB1*1303
	RR	DRB45	DRB1*1001
	F--RR-E	DRB62	DRB1*0901
20	I--A	DRB63	DRB1*1501, 1502; DRB5*0201, 0202
	Y	DRB103	DRB1*0301, DRB3*0101
	E	DRB102	DRB1*110X
	S	DRB60	DRB1*0405, 0801, 0803, 1303
	A--H	DRB112	DRB1*1401, LY10
25	V--S	DRB35	DRB1*070X, 0901, 1201; DRB3*0101, 0301
	R	---	DRB1*0101, 0102, 0404, 0405, 0408, 1402
	K	---	DRB1*0401
30	R--E	---	DRB1*0403, 0406, 0407
	RR--E	---	DRB1*1401, LY10; DRB4*0101
	I--DR	DRB72	DRB1*0701, 0702
	I--DR	DRB95	DRB1*0803, 1201
	K--GR	---	DRB1*0301, 0302; DRB3*0101
35	DR	DRB113	DRB1*1602
	G (pos. 86)	---	See Table 5
	V (pos. 86)	---	See Table 5

<u>Epitope</u>	<u>Name</u>	<u>Specificity</u>
AV (pos. 86)	---	DRB1*0102, 1201; DRB5
WNIIN	GH51	DRB4*0101
IHKR	DRB101	DRB2*0101

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Example 9Reverse Dot-Blot Typing Kit

HLA DRB typing kits incorporating a rapid and simple reverse dot blot hybridization format were designed to provide a simple and fast prescreening of samples before proceeding to fine subtyping involving allele specific amplification.

- 10 The kits contain the amplification reagents, DNA for use as a positive control, nylon strips on which the probes have been immobilized, colorimetric detection reagents, reaction tubes, and instructions.

- 15 Typing was carried out using two amplification reactions, one using DRB general primers and the other using DRB1 specific primers. The primer pairs amplify under the same thermocycler conditions so that the two reactions could be carried out concurrently. Two panels of probes, one specific for each amplification reaction, were used in the reverse dot blot hybridization format. Each panel of probes was immobilized on a single nylon strip for ease of handling.

- 20 Biotinylated primers were used in the amplification reactions to allow for later detection using a colorimetric assay as described in Example 8, above. The DRB amplification primers used were CRX28 (SEQ ID NO: 67) and CRX29 (SEQ ID NO: 68). The DRB1 amplification primers were CRX28 and CRX37 (SEQ ID NO: 73). Both sets of primers are described in Example 8, above.

- 25 The two probe panels, one for hybridizing with the amplification products of the DRB amplification and the other for hybridizing with the amplification product from the DRB1-specific amplification, are shown below. The nucleotide sequence for each probe is found in the Sequence Listing section; the SEQ ID NO: for each probe is provided below.

Probe Panel for the DRB Amplification

	<u>Probe</u>	<u>Seq. ID No.</u>	<u>AA Sequence</u>	<u>Reactivity</u>
	1 DRB01	SEQ ID NO: 79	WLF	DR1
	2 GH104	SEQ ID NO: 90	WPR	DR2
5	3 DRB46	SEQ ID NO: 123	YSTS	DR3, 11, 13, 14
	4 DRB48	SEQ ID NO: 125	V-H	DR4
	5 DRB207	SEQ ID NO: 283	G-YK	DR7
	6 GH102	SEQ ID NO: 89	YSTG	DR8, 12, 1404
	7 DRB209	SEQ ID NO: 285	K-D-F	DR9
10	8 DRB20	SEQ ID NO: 98	EV	DR10
	9 DRB102	SEQ ID NO: 178	E	DR11
	10 DRB112	SEQ ID NO: 188	A-H	1401, 1404
	11 DRB07	SEQ ID NO: 84	F-DE	1103
	C DRB42	SEQ ID NO: 119	TELGRP	ALL

15

Probe Panel for the DRB1 Amplification

	<u>Probe</u>	<u>Seq. ID No.</u>	<u>AA Sequence</u>	<u>Reactivity</u>
	12 DRB223	SEQ ID NO: 299	S	*
	13 DRB37	SEQ ID NO: 114	F-DR	*
	14 DRB203	SEQ ID NO: 279	R	*
20	15 DRB163	SEQ ID NO: 239	K	0401, 0409
	16 DRB118	SEQ ID NO: 194	R-E/RR-E	*
	17 DRB02	SEQ ID NO: 61	I-DE	*
	18 DRB38	SEQ ID NO: 115	K-GR	DR3
	19 DRB222	SEQ ID NO: 298	Y	0301
25	20 DRB232	SEQ ID NO: 308	I-DK	1303
	21 DRB136	SEQ ID NO: 212	I-DR	0803, 1201 (not DR7)
	22 DRB198	SEQ ID NO: 274	V-S	DR7, 0803, 1201
	C DRB42	SEQ ID NO: 119	TELGRP	ALL

* sequence is found on a number of different alleles (see DRB1 amino acid alignment).

30

DRB probes 1-8 are specific to the region about amino acids 9-13; probe 9 is specific for amino acid 58; probe 10 is specific for amino acids 57-60; and probe 11 is specific for amino acids 67-74. DRB1 probes 13-18, 20, and 21 are specific to amino acids 67-74; probe 12 is specific to amino acid 57; probe 20 is specific to amino acid 26; and probe 22 is specific for amino acids 57-60. The control probe is specific for

35

amino acids 51-56.

It should be noted that the probes shown in the above panels can also be labeled and used in the DRB typing methods described in Example 7. The same hybridization and wash conditions would be used. Interpretation of the hybridization pattern would be as described below.

5 The kits were packaged as two boxes: one box contained the DRB reagents and the other box contained the DRB1 reagents. Packaged in each box was either DRB or DRB1 PCR mix, DNA control, and typing strips; 8 mM magnesium chloride solution; mineral oil; SA-HRP conjugate; chromogen (TMB); reaction tubes; and instructions. PCR contains the reagents necessary for a PCR with the exception of the magnesium
10 chloride and the template DNA. Other reagents and equipment needed to perform the methods described were supplied by the kit user; all are commonly available commercially.

Several sample preparation procedures suitable for use in PCR amplifications are known in the art. A preferred procedure is the Chelex extraction method described
15 in Singer-Sam *et al.*, 1989, Amplifications 3:11, and Walsh *et al.*, 1991, BioTechniques 10(4):506-513, both of which are incorporated herein by reference. For examples of other techniques for extracting nucleic acids from biological samples, see those described in Sambrook *et al.*, Molecular Cloning: A Laboratory Manual (New York, Cold Spring Harbor Laboratory, 1989); Arrand, Preparation of Nucleic
20 Acid Probes, in pp. 18-30, Nucleic Acid Hybridization: A Practical Approach (Ed Hames and Higgins, IRL Press, 1985); or, in PCR Protocols, Chapters 18-20 (Innis *et al.*, ed., Academic Press, 1990), which are all incorporated herein by reference.

Primer pairs for amplifying all DRB sequences and for amplifying specifically DRB1 sequences are used in two separate PCR reactions. Amplification reactions are
25 essentially as described in Saiki *et al.*, 1989, Proc. Natl. Acad. Sci. USA 86:6230-6234, incorporated herein by reference, with the modifications described below. Reactions are carried out using 25 μ l of sample in a total volume of 100 μ l, each reaction containing 50 mM KCL, 10 mM TrisHCl (pH 8.4), 1.5 mM $MgCl_2$, 10 μ g Gelatin, 200 μ M each dATP, dCTP, dGTP, and dTTP, 0.2 μ M each biotinylated
30 amplification primer, and 2.5 units of *Thermus aquaticus* DNA polymerase (PECI).

The same temperature profile is used for both amplification reaction, allowing both reaction to be carried out concurrently in the same thermocycler. The thermocycler is programmed for 35 cycles of the following temperature profile: denature at 95°C for 60 seconds, anneal at 60°C for 30 seconds, extend at 72°C for 60 seconds. The
35 thermocycler is programmed to incubate the sample for an additional 7 minutes at 72°C following the last cycle.

The amplified DNA from the PCR is in double stranded form and must be denatured to allow hybridization with the oligonucleotide probes. The amplified DNA is denatured at 95°C for 5 to 10 minutes in the thermocycler and maintained at that temperature until used. Alternatively, amplified DNA which has been denatured can be transferred directly from the 95°C temperature into an ice bath. The rapid cooling stabilizes the DNA in a denatured form. The amplified DNA is then kept in the ice bath until it is required for the hybridization.

Twelve probes are affixed to each of two nylon membrane strips; one strip contains the probes to be used for hybridization with the DRB amplification product and the other strip contains the probes for hybridization with the DRB1-specific amplification product. Hybridization reactions are carried out in trays which hold each probe-containing strip in a separate well. The hybridization trays are commercially available from PECO and are typically supplied with the kit. The hybridization conditions described below are specific for the DRB hybridization. The DRB1 DNA hybridization protocol differs from the DRB protocol in that the DRB1 hybridization is at 55°C instead of the 50°C. All other aspects of the protocol are identical.

In each well containing a strip, 3 ml of pre-warmed hybridization solution (4X SSPE and 0.5% weight/volume SDS) are added followed by 25 µl of amplified DNA. After careful mixing of the tray contents, the tray is placed in a 50°C shaking water bath and incubated at 50°C for 20 minutes at about 50 rpm.

The strips are washed initially in 10 ml of wash solution (1.0X SSPE and 0.1% weight/volume SDS) at room temperature for several seconds prior to the stringent wash and each well is then aspirated. The temperature and timing of the stringent wash are critical. Pre-warmed wash solution (10 ml) are added to each well and the tray is incubated in a 50°C shaking water bath for 12 (± 2) minutes at about 50 rpm. After aspirating each well, 10 ml of wash solution are added to each well, the tray is incubated at room temperature for 5 minutes on an orbital shaker at about 50 rpm, and the wells are again aspirated.

To each well containing a strip are added 3 ml of wash/enzyme conjugate solution (from a solution of 3.3 ml of wash solution and 27 µl of enzyme conjugate prepared within 15 minutes of use). The tray is incubated at room temperature for 20 minutes on an orbital shaker at approximately 50 rpm. The solution is aspirated from each well and the strips are washed in 10 ml of wash solution at room temperature for 5 minutes on an orbital shaker at approximately 50 rpm. Finally, the wash solution is aspirated from each well and the strips are ready for the color development step.

The color development steps are identical for both DRB and DRB1. To each well is added 10 ml of citrate buffer (100 mM Sodium Citrate, pH = 5.0) and the tray is